# *In vitro*, *in vivo* and *in ovo* interactions of human pathogenic fungus *Aspergillus fumigatus*.

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For my parents

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**Summary**

*Aspergillus* (*A*.) *fumigatus* is an opportunistic pathogenic fungus which is one of the commonly causes of invasive aspergillosis in humans with compromised immunity. The interplay of *A. fumigatus* with its host often determines the severity of the disease and hence this host-pathogen interaction is evaluated at different levels e.g., at organism level and molecular level. However, various aspects of *A. fumigatus* interactions with its host remain elusive and a better understanding of fungal pathogenesis is required. Therefore, during this study, the following challenges were overcome:

1) **Evaluating the role of conidial melanin on interaction of *A. fumigatus* with alveolar epithelial cells:**

Dihydroxynaphthalene (DHN) melanin present on the conidial surface of *A. fumigatus* is an important virulent factor and protects the fungus from various host immune attacks. The first encounter of invading conidia is with epithelial cells and the role of melanin was elucidated on interaction of conidia with these cells. It was revealed that *A. fumigatus* conidia are able to survive and reside inside alveolar epithelial cells and melanin plays an essential role in its survival. Moreover, by using melanin mutant strains, it was found that presence of melanin plays a fundamental role in the uptake of conidia by epithelial cells. Additionally, it was demonstrated that melanin is able to inhibit apoptosis or cell death and this effect increased with increase in internalization of conidia by epithelial cells.

2) **Elucidating a mouse model for dissemination of *A. fumigatus* infection based on pulmonary infection.**

*A. fumigatus* is able to cause various forms of disease which mainly occur in lungs due to inhalation of airborne conidia and is also able to cause systemic infections in severely immunocompromised patients. Though various models are used to study *A. fumigatus* host-pathogen interactions, however, no adequate model has been established to investigate the dissemination of fungus to other organs based on the pulmonary infection. Mouse was, therefore, used as a model to follow the spread of infection after inhalation of conidia. The data presented in this study reveal that in immunocompetent mice, the fungus was cleared within 24 hours after pulmonary infection and there was no presence of fungus in other organs. In immunocompromised mice, there was presence of fungal DNA in brain in addition

to lungs but the fungus was not found viable. No clear evidence could be provided for establishment of mice as model for dissemination of infection after pulmonary infection.

**3) Establishing the test system for evaluation of novel radiotracers for diagnosis of *A. fumigatus* infections using chicken embryo model and Ligand Tracer instrument.**

Positrom Emission Tomography (PET) combined with Computed Tomography (CT) is one of the promising diagnostic tools for *A. fumigatus* infections and requires the use of radiotracers. These radiotracers are developed and characterized using various assays and engage different animal models. Therefore, this study revealed the characterization of radiotracers using chicken embryos for PET/CT imaging and ligand tracer technology for *in vitro* assaying of radiotracer. It was demonstrated that ligand tracer can be optimized for evaluation of novel radiotracers for *A. fumigatus* infections. Furthermore, using desferri-triacetylfusarinine C (TAFC) as proof of principle, it was shown that chicken eggs can serve as model for characterization of novel radiotracers for *A. fumigatus* infections. Both the models together can be used for initial characterization of radiotracers for diagnosis of *A. fumigatus* infections.

In summary, this study provides a deeper understanding on interactions of *A. fumigatus* with host on organism level (mouse and chicken embryos) as well as on molecular level (alveolar epithelial cells).

# Zusammenfassung

*Aspergillus (A.) fumigatus* ist ein opportunistischer pathogener Pilz und eine der häufigsten Ursachen für invasive Aspergillose in Menschen mit Immunschwäche. Die Wechselwirkung von *A. fumigatus* mit seinem Wirt bestimmt oft die Schwere der Erkrankung und daher wird diese Wirts-Pathogen Interaktion auf verschiedenen Ebenen wie der Organismen- und der molekularen Ebene untersucht. Dennoch ist über verschiedene Aspekte der Interaktion von *A. fumigatus* mit seinem Wirt wenig bekannt, was ein besseres Verständnis der pilzlichen Pathogenese erfordert. Deshalb wurden in der vorliegenden Arbeit folgende Problemstellungen untersucht:

1) **Untersuchung der Rolle von conidialem Melanin bei der Interaktion von *A. fumigatus* mit alveolaren Epithelzellen:**

Das auf der Oberfläche der Conidien von *A. fumigatus* befindliche Dihydroxynaphthalen (DHN) ist ein bedeutender Virulenzfaktor und schützt den Pilz vor verschiedenen Attacken des Immunsystems des Wirtes. Conidien treffen bei ihrer Invasion des Wirtes zunächst auf Epithelzellen. Deshalb wurde die Rolle von Melanin bei der Interaktion von Conidien mit diesen Zellen untersucht. Es wurde gefunden, dass Conidien von *A. fumigatus* in alveolaren Epithelzellen überleben und existieren können und dass Melanin eine entscheidende Rolle in diesem Überleben spielt. Mehr noch, durch die Verwendung von Melanin-Mutantenstämmen wurde gefunden, dass die Anwesenheit von Melanin eine fundamentale Rolle bei der Aufnahme von Conidien durch Epithelzellen spielt. Weiterhin wurde demonstriert, dass Melanin in der Lage ist Apoptose zu inhibieren und dieser Effekt verstärkt wird durch eine gesteigerte Internalisierung von Conidien durch Epithelzellen.

2) **Beschreibung eines Mausmodells für die Dissemination von *A. fumigatus* Infektionen basierend auf pulmonaler Infektion:**

*A. fumigatus* kann verschiedene Krankheitsbilder verursachen, die hauptsächlich in der Lunge nach Inhalation von Conidien aus der Luft ablaufen, kann aber auch systemische Infektionen in schwer Immun-geschwächten Patienten hervorrufen. Obwohl verschiedene Tiermodelle genutzt werden um die Wechselwirkung von *A. fumigatus* mit seinem Wirt zu

untersuchen, gibt es keine adequaten Modelle um die Dissemination des Pilzes in andere Organe nach pulmonaler Infektion zu untersuchen. Deshalb wurde am Mausmodell die Verbreitung der Infektion nach Inhalierung der Conidien untersucht. Die Daten der vorliegenden Arbeit zeigen, dass in Immun-kompetenten Mäusen der Pilz 24 Stunden nach pulmonaler Infektion eleminiert ist und in anderen Organen nicht nachgewiesen werden kann. In Immun-geschwächten Mäusen wurde pilzliche DNA im Gehirn zusätzlich zur Lunge gefunden, allerdings war der Pilz nicht lebensfähig. Somit konnte kein endgültiges Mausmodell, um die Dissemination des Pilzes in andere Organe nach pulmonaler Infektion zu untersuchen, präsentiert werden.

**3) Etablierung eines Testsystems zur Evaluierung von neuen radioaktiven Tracern für die Diagnostik von *A. fumigatus* Infektionen unter Nutzung des Hühnerembryonen-Modells und eines Ligand Tracer Gerätes**

Positronen Emissions-Tomographie (PET) kombiniert mit Computer-Tomographie (CT) ist eine der vielversprechendsden Diagnostik-Methoden für *A. fumigatus* Infektionen und erfordert den Einsatz von radioaktiven Tracern. Diese Tracer werden entwickelt und charakterisiert unter Nutzung verschiedener Assays und Tiermodelle. Deshalb wurde in der vorliegenden Arbeit unter Nutzung des Hühner-Embryonenmodells radioaktive Tracer für das PET/CT-Imaging und für die Ligand Tracer Technologie zum *in vitro* Assay der Tracer getestet. Es wurde die Eignung der Ligand Tracer Technologie für die Detektierung von *A. fumigatus* Infektionen gezeigt. Darüberhinaus wurde mit Desferritriacetylfusarinin C (TAFC) als Modell gezeigt, dass Hühnereier als Modell für die Charakterisierung neuer radioaktiver Tracer für die Detektierung von *A. fumigatus* Infektionen dienen können. Beide Modelle zusammen können für die initiale Charakterisierung von radioaktiven Tracern für die Diagnose von *A. fumigatus* Infektionen genutzt werden.

Alles in allem liefert die vorliegende Arbeit ein vertieftes Verständnis der Interaktion von *A. fumigatus* mit dem Wirt sowohl auf der Organismenebene (Mausmodelle und Hühner-Embryonenmodell) als auch der molekularen Ebene (alveolare Epithelzellen).

# 1 Introduction

## 1.1 The opportunistic human pathogen *Aspergillus fumigatus*

The saprophytic fungus *Aspergillus* (*A*.) *fumigatus* is ubiquitously found in nature in dead and decaying organic matter, compost heap and indoor air environment (Tekaia and Latge, 2005), where it plays an essential role in carbon and nitrogen recycling (Latge, 2001). The ascomycetous, filamentous fungus *A. fumigatus*, during asexual sporulation, involves the formation of uninucleate and haploid spores, called conidia (*singular* conidium). These conidia grow on specialized reproductive apparatus known as conidiophores and are produced in chains by flask-shaped greenish phialides attached to conidiophores (Fig 1A) (Brakhage and Langfelder 2002; Latge 2001). The conidial cell wall is a physically rigid layer and helps in protection of fungal cell from its environment (Bernard and Latge, 2001). The outermost cell wall layer of *A. fumigatus* conidia, known as rodlet layer, is composed of interwoven rodlet fascicles composed of hydrophobic proteins. This layer is known to give hydrophobic properties to *A. fumigatus* conidia (Thau N et al., 1994; Paris et al., 2003). Adjacent to the rodlet layer is a dense pigmented outer layer, consisting of dihydroxynaphthalene (DHN)-melanin, and a translucent inner layer (Fig 1B) (Langfelder et al., 2003). Different polysaccharides like, β-(1-3) glucans, β-(1-6) glucans, galactomannan and chitin are covalently interconnected and form the central core of the cell wall (Gastebois et al., 2009; Latge 2007). Resting conidia can remain viable for months due to their metabolically quiescent nature. The first step of development of new colonies is swelling of the conidia which starts within hours of arriving into a feasible environment and is followed by germination of conidia and subsequent elongation of hyphae (Park and Mehrad, 2009). The process of germination mostly involves changes in the ultrastructure of conidial wall and requires cell wall softening which is acquired by hydrolysis of polysaccharides and is accompanied by the shedding of the rodlet layer and the synthesis of a new cell wall layer which originates from translucent inner layer (Latge et al. 2005). The grey-green conidia with diameter of about 2-3 µm readily become airborne and get dispersed widely under a broad range of environmental conditions (Latge, 1999). According to different studies, it is estimated that the mean concentration of *A. fumigatus* conidia in air ranges from 0.2 – 15 conidia/$m^3$ up to $10^6$ conidia/$m^3$ in some agricultural settings (VandenBerg et al., 1999).

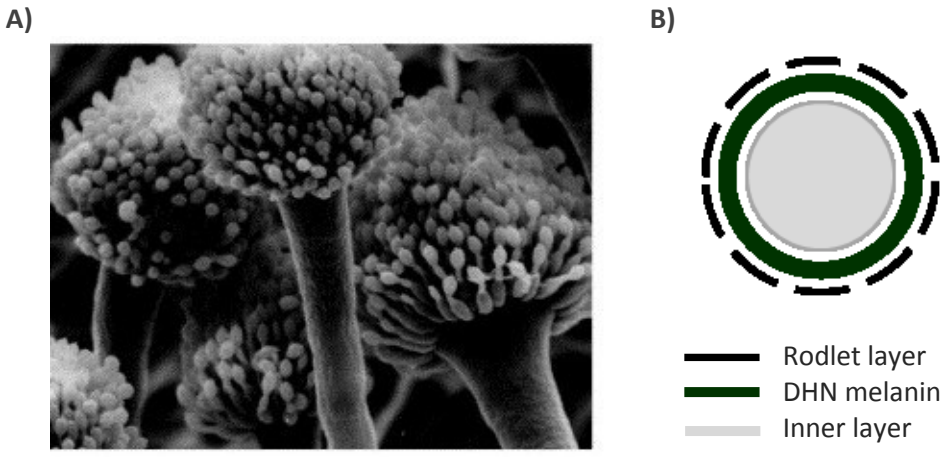



**Figure 1: Morphology of *A. fumigatus***
**A) The electron micrograph of *A. fumigatus* conidiophores** (Latge, 2001). During asexual cycle, the haploid hyphae form foot cells which develop into conidiophores bearing phialides. The phialides form long chains comprising of conidia that are released into atmosphere. **B) Conidial cell wall structure**. The inner most layer of conidia is a double layered cell wall with outer dense pigment layer made of DHN melanin and inner translucent electron layer which consists of branched glucans linked to chitin. The outer most layer is made up of interwoven rodlet fascicles, regularly arranged, known as rodlet layer.

### 1.1.1 Medical importance of *A. fumigatus*

*A. fumigatus* is the main causative agent of human diseases among the human pathogenic species of *Aspergillus* (Denning DW, 1998; Morgan et al., 2005). *A. fumigatus* conidia are able to germinate at temperatures of up to 60 °C in contrast to other fungal species (Araujo and Rodrigues, 2004) and can maintain its survival at temperatures up to 70 °C (Latge, 1999). Vertebrate endothermy was speculated to have evolved primarily to protect against fungal infections (Casadevall, 2005). Due to thermophilic nature of *A. fumigatus* conidia over other species (Beffa 1998, Bhabhra and Askew 2005), it is capable of infecting mammalian hosts and thrives inside human body due to constant exposure of humans to these airborne conidia. It is estimated that humans inhale several hundred *A. fumigatus* conidia per day. In healthy individuals, the conidia are efficiently cleared, however, in humans suffering from an impaired immune system, the conidia can germinate and thereby cause severe infections (for an overview see (Brakhage, 2005; Dagenais and Keller, 2009)). In normal immune-competent individuals, the infectious conidia are efficiently eliminated by host immune system, though some allergic reactions such as sinusitis and bronchoalveolitis can occur (Latge, 1999; Schubert, 2009). However, in immunosuppressed patients involving chemotherapies, organ transplants and various immunosuppressive agents, this pathogenic

fungus causes severe and fatal invasive infections (Denning DW, 1998; Dixon et al., 1996; Groll AH et al., 1996). The diseases caused by *A. fumigatus* can be categorized into three main types: i) Non-invasive aspergilloma or fungal ball, in which *A. fumigatus* colonises the lungs or the sinuses with restricted invasiveness (Ellis, 1999), ii) Allergic reactions, such as, bronchopulmonary aspergillosis, associated in individuals with altered lung functions, like asthma, allergic sinusitis and cystic fibrosis. These allergic reactions are associated with no mycelia growth. iii) Systemic infections, such as, invasive aspergillosis (IA) which is one of the most fatal *Aspergillus*-related diseases (Dagenais and Keller, 2009), targeting patients such as those with acute leukaemia (Pagano 2001), HIV infection (Feldmesser 2005), corticosteroid therapies (Palmer et al 1991), organ and hematopoietic stem cell transplant recipients (Marr et al., 2002; Mikulska et al, 2009; Post et al., 2007; Brakhage 2005), or chronic granulomatous disease (CGD) (Almyroudis et al, 2005; Segal and Romani, 2009). Four types of invasive aspergillosis (IA) have been described so far (Denning DW, 1998; Verweij PE and Denning DW, 1997): a) Chronic or acute pulmonary aspergillosis which is the most common form of IA. b) Obstructive bronchial disease or tracheobronchitis (Denning DW et al., 1994; Kemper et al., 1993; Nash G, 1997) which is mostly seen in AIDS patients. c) Acute invasive rhinosinustis (Drakos et al., 1993; Morgan MA et al., 1984; Viollier AF, 1986; Washburn RG 1988). d) Disseminated disease which commonly involves the dissemination of fungus to brain and other organs (Bodey G et al., 1992; Pagano L et al., 1996, Ribaud P et al., 1999; Wingard et al., 1987). Depending on the immunological status of the host, the mortality caused by invasive aspergillosis still remains high, ranging from 30 – 90% (Brakhage 2005).

### 1.2 Innate immune response against *A. fumigatus*

Inhalation of conidia is the primary route of *A. fumigatus* infection in humans. Due to small size and airborne nature of conidia, they get deeply deposited in the alveoli of lungs and immediately encounter the innate defence responses in immunocompetent individuals which play an essential role in clearance of fungus in these individuals (Schaffner A, 1989; Schaffner A, 1982; Schaffner A, 1994). In nonspecific innate immune responses, anatomical barriers form the first major line of defence which mainly comprise of mucous membrane and mucociliated cells. In addition, phagocytic cells such as alveolar macrophages,

neutrophils and dendritic cells as well as humoral components such as complement system form the other major lines of defence of innate immunity. Majority of inhaled conidia are cleared by the ciliary action of mucous epithelium, however, the conidia that escape the mucociliary clearance are encountered by epithelial and phagocytic cells (Fig 2).

Phagocytosis, besides other mechanisms, forms the main effector mechanism against invading fungal pathogens which is mediated not only by professional phagocytic cells like alveolar macrophages but also by non-professional phagocytes like repiratory epithelial cells. Alveolar macrophages, the most prominent professional phagocytes in alveoli of lungs, phagocytose conidia and kill the ingested conidia within phagolysosomes (Ibrahim-Granet O, 2003). Macrophages secrete various cytokines and chemokines which help in recruitment of other phagocytic cells like neutrophils and dendritic cells to target and kill the germinating hyphae and ingested conidia, respectively. There are no reports about 100% killing of inhaled *A. fumigatus* conidia by alveolar macrophages (Latge 1999). Also, conidia germinate and form mycelia which suggest the role of neutrophil in killing these germinating conidia and mycelia. Neutrophils form neutrophil extracellular traps (NETs) to attack the growing conidia and mycelia (Bruns et al., 2010)

The initial step in the process of phagocytosis involves the binding of receptors on the phagocytic cell surface to microbial cell surface components. Recognition of *A. fumigatus* conidia by macrophages occurs after shedding the hydrophobic rodlet layer during swelling and germination (Brakhage 2010, Brown et al., 2002). This exposes the underlying pathogen-associated molecular patterns (PAMPs) such as, β-(1,3)-glucan which are recognized by pattern recognition receptotrs (PRRs) present on phagocytic cells of the host. Recognition of swollen conidia and hyphae involves C-type lectin receptor dectin-1 and Toll like receptors (TLR) such as TLR 2, 4 and 9 (Steele et al., 2005; Luther et al, 2007; Ramirez-Ortiz et al., 2008), however, recognition and phagocytosis of resting conidia, that humans inhale, have not been elucidated in detail so far. Resting conidia that reach the lung alveoli are inert due to presence of rodlet layer and melanin layer present on the conidial cell wall (Aimanianda, V., et al., 2009).

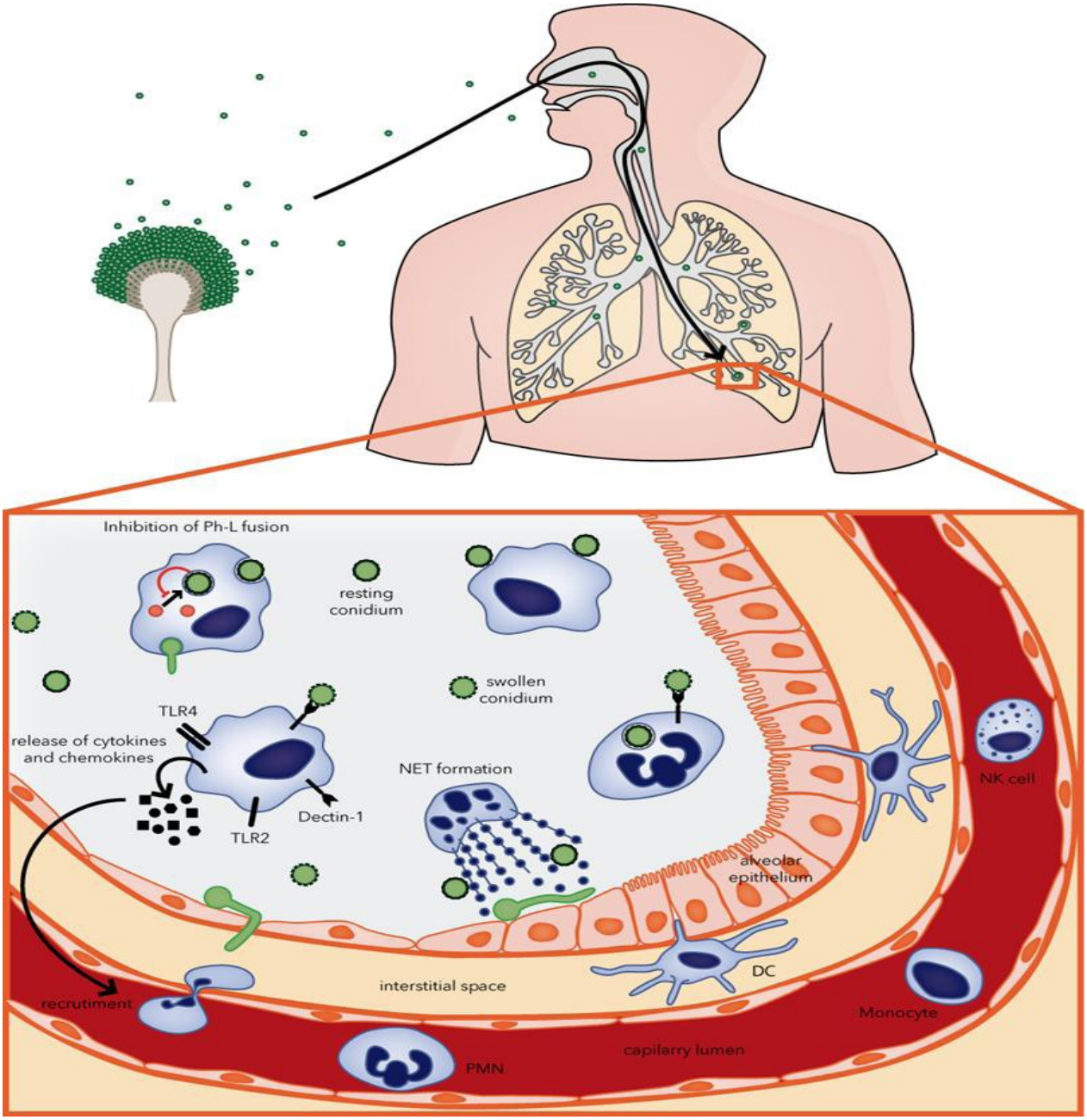



**Figure 2: Innate immune response to inhaled *A. fumigatus* conidia**
*A. fumigatus* conidia are airborne and the primary route of infection is via inhalation of airborne conidia. Due to small size, the conidia are able to reach deep inside the alveoli of lungs and encounter the host immune response. The epithelial cells form the first barrier to invading conidia and phagocytose these conidia. The conidia which escape from alveolar epithelium are taken up by other immune cells which form the main host defence against invading conidia. Alveolar macrophages phagocytose resting and swollen conidia and recruit other immune cells like neutrophils and dendritic cells via secretion of cytokines and chemokines. Neutrophils attack conidia, germinating conidia and mycelia by forming neutrophil extracellular traps (NETs) while as dendritic cells engulf conidia and help in initiating the adaptive immune response (Adapted from Volling, K., dissertation; Brakhage, 2010).

### 1.2.1 Role of pulmonary epithelial cells in innate immune response

The airway epithelium, besides being merely a physical barrier against invading respiratory micro organisms, acts as a frontline defense with its mucociliary action and immunological functions. Once *A. fumigatus* conidia are inhaled, they first encounter respiratory epithelia and subsequently reach the alveolar epithelial cells which likely contribute to overall immune response to the fungus (Dagenais and Keller, 2009). Alveolar epithelial cells interact with alveolar macrophages for initiation of immunological host defences against inhaled *A. fumigatus* conidia. The conidia rapidly adhere to pulmonary epithelial cells (Hope et al., 2007, DeHart, DJ., et al., 1997, Paris, S., et al., 1997) and are taken up by these cells. The internalization of conidia by epithelial cells results in the formation of membrane blebs and subsequent loss of actin fibers. Epithelial cells recruit different mechanisms against invading conidia such as, secretion of antimicrobial peptides or defensins that play a role in airway defense (Alekseeva et al., 2009) and production of proinflammatory cytokines and chemokines e.g., IL-6, TNF-α and IL-8 (Fig 3) (Balloy et al., 2008; Bellanger et al., 2009). Chemokines like, IL-8 contribute to effective recruitment and activation of neutrophils at the site of infection (Fig 3) (Mukaida 2000; Pease and Sabroe 2002).

Alveolar epithelial cells are able to internalize *A. fumigatus* conidia where they enter the acidic phagolysosomes and are killed. Some conidia are able to escape the phagolyosomal acidification without causing any damage to the cells (Walysnka and Moore 2002; Walysnka and Moore 2003). These conidia germinate into mycelium and invade towards the extracellular environment. Several conidial factors such as sialic acid residues contribute to binding and uptake of fungus by epithelial cells (Bromley and Donaldson, 1996; DeHart et al, 1997). However, besides sialic acid, other unknown factors are suggested to be involved in binding to alveolar components, thus contributing to overall pathogenicity of the fungus (Dagenais and Keller, 2009). Furthermore, epithelial cells besides being the first cellular line of interaction between fungus and the host, and are supposed to play a role for the progress and development of a fungal infection (Filler and Sheppard, 2006), to date, there is limited information about the interplay between epithelial cells and conidia, in particular when recognition and intracellular processing of conidia are concerned.

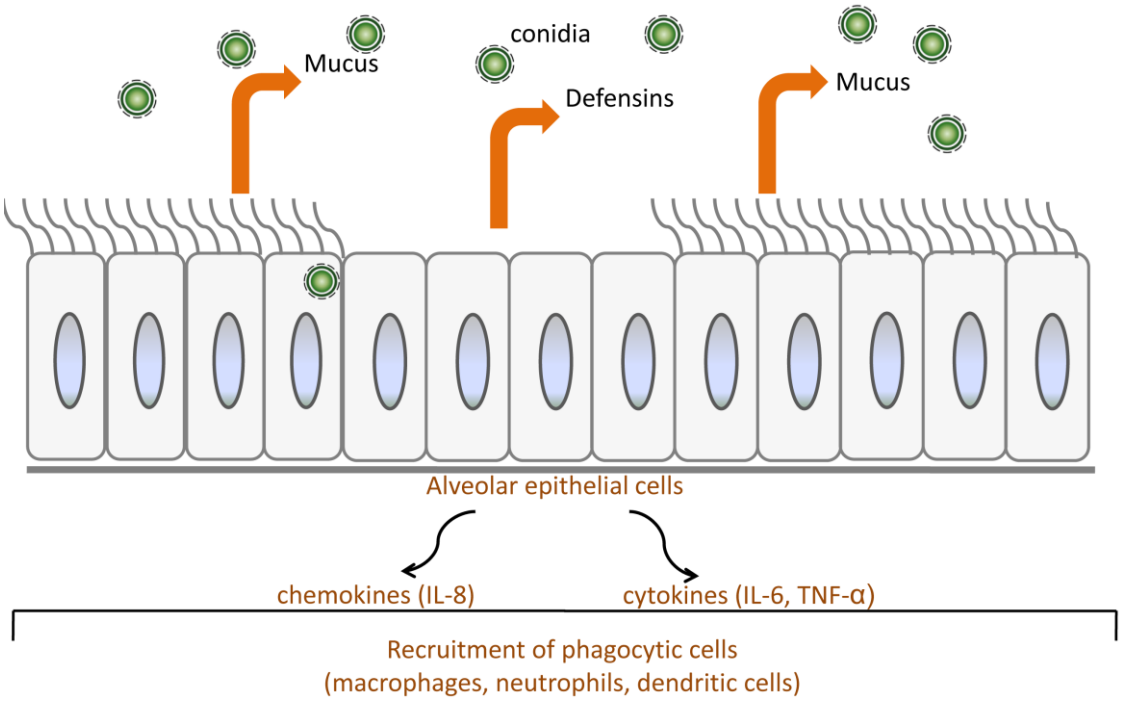



**Figure 3: Interaction of *A. fumigatus* with respiratory epithelium**
Upon inhalation of airborne conidia, the airway epithelia encounter the invading conidia and phagocytose them. Epithelial cells secrete soluble antimicrobial compounds that are important for airway defence. Secretion of cytokines and chemokines by epithelial cells assist in initiation of proinflammatory response against conidia and recruit the immune cells such as alveolar macrophages.

## 1.3 Virulence determinants of *A. fumigatus*

*A. fumigatus*, capable of surviving in different conditions, such as, in dead and decaying matter, causes infections in immune suppressive individuals and is adapted to various physical, biological and chemical stresses prevailing around the organism. *A. fumigatus* mediate various defences that contribute to its pathogenicity (Askew, D.S, 2008; Rhodes, J.C, 2006) and are multifactorial. *A. fumigatus* mediated defence mechanisms include various factors, such as, thermotolerance and production of secondary metabolites and enzymes, the fungal cell wall, small size of conidia and production of melanin (Alp, S. and Arikan, S., 2008). The production of pigment is one of the factors that contribute to pathogenicity and protects fungus against various stresses.

### 1.3.1 Melanin

Melanin is a negatively charged hydrophobic pigment and mostly black or brown in colour (Gomez, B.L and Nosanchuk, J.D., 2003, Jacobson, E.S., 2000; Riley, P.A., 1997). Melanin gives a survival advantage to the fungus by providing protection against enzymatic lysis, oxidative stress, UV and solar radiations, extreme temperatures and heavy metals and helps in combating various stresses in its heterogenous environments (Langfelder et al., 2003). Melanin has been shown to scavenge the free electrons from reactive oxygen species (ROS) (Commoner et al., 1954: Liu, G.Y., and Nizet, V., 2009), modulate inflammatory response (Huffnagle et al., 1995), interfere with antifungal peptides, such as, Amphotericin B (Van Duin et al., 2002) and also shown to interfere with macrophage function (Mednick et al., 2005, Wang et al., 1995, Volling et al., 2011).

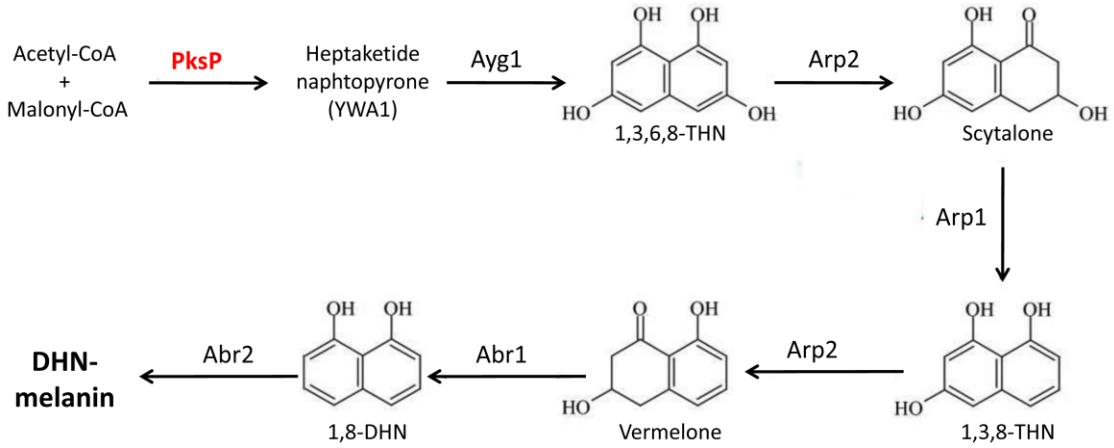



**Figure 4: DHN-melanin biosynthesis pathway**
Various enzymes are involved in the biosynthesis of DHN melanin. The first enzyme is PksP (polyketide synthase). Disruption of PksP leads to formation of white conidia which lack DHN-melanin layer. The other enzymes involved are Ayg (*Aspergillus* yellowish-green), Arp (*Aspergillus* reddish-pink1) and Arb (*Aspergillus* brown) and the disruption of these enzymes lead to yellowish-green, reddish-pink and brown conidia, respectively.

The grey green color of *A. fumigatus* conidia is due to the presence of dihydroxynaphthalene (DHN) melanin synthesized from acetyl Co A and malonyl Co A in the presence of various genes (Bernard & Latge 2001, Latge 2001, Tsai et al.,1998). Among these cluster of genes, polykitide synthase pksP (or Alb 1) catalyzes the first step in melanin pathway (Fig 4). Deletion of this gene produces smooth walled, pigmentless white conidia and are shown to be less pathogenic in murine model of invasive aspergillosis (Langfelder et al., 1998), inhibit

phagolysosomal fusion (Jahn et al., 2002) and are susceptible to various damages caused by macrophages (Jahn et al., 1997). Taken together, melanin contributes to the overall pathogenicity of *A. fumigatus* but pathogenecity seems to be dependent on a network of various molecular and physiological properties, due to the fact *that A. nidulans* also synthesizes melanin and is non-pathogenic in nature.

### 1.4 Apoptosis

Cell death is generally divided in two sub groups, unregulated and regulated cell death. Unregulated, also known as necrosis cell death lacks the organized events and results from direct damage of cells. Unlike necrosis, apoptosis is a regulatory physiological cell death, a vital process which is essential for organism development, tissue homeostasis and also helps to get rid of damaged cells (Jacobsen et al., 1997, Meier et al., 2000). Additionally, apoptosis is a key process in host innate immunity, especially in the defence mechanisms against microbial infectons (Bellamy et al., 1995, Williams 1994). This programmed cell death is triggered extracellularly or intracellularly by two major signaling pathways, *i.e*., the extrinsic or death receptor mediated pathway which is initiated by members of tumor necrosis factor (TNF) superfamily, and the intrinsic or mitochondrial mediated pathway which is initiated by various stimuli such as DNA damage, ROS, gamma irradiation or cytotoxic drugs (Fig 5). Both pathways involve the activation of aspartate specific cysteine proteases, known as caspases (Martinou and Green, 2001, Thornberry and Lazebnik 1998), which are responsible for biochemical and morphological changes associated with apoptosis (Taylor et al., 2008). These changes include DNA fragmentation, chromatin condensation, cell shrinkage and formation of apoptotic bodies which are efficiently cleared by phagocytes without any inflammatory response (Savill 2000). Caspases are present as inactive precursors, known as pro-caspases and are activated upon proteolytic cleavage. Caspases are classified as initiator (8, 9) and executioner caspases (or effector caspases such as 3, 6, and 7) (Thornberry and Lazebnik 1998). The former caspases translate the apoptotic signal to caspase cascade and the later caspases are responsible for cleaving the apoptitic relevant substrates. Caspase-3 is a central element of both apoptotic pathways. However, a caspase-independent apoptotic pathway has also been reported (Chose et al., 2003; Susin et al., 2000).

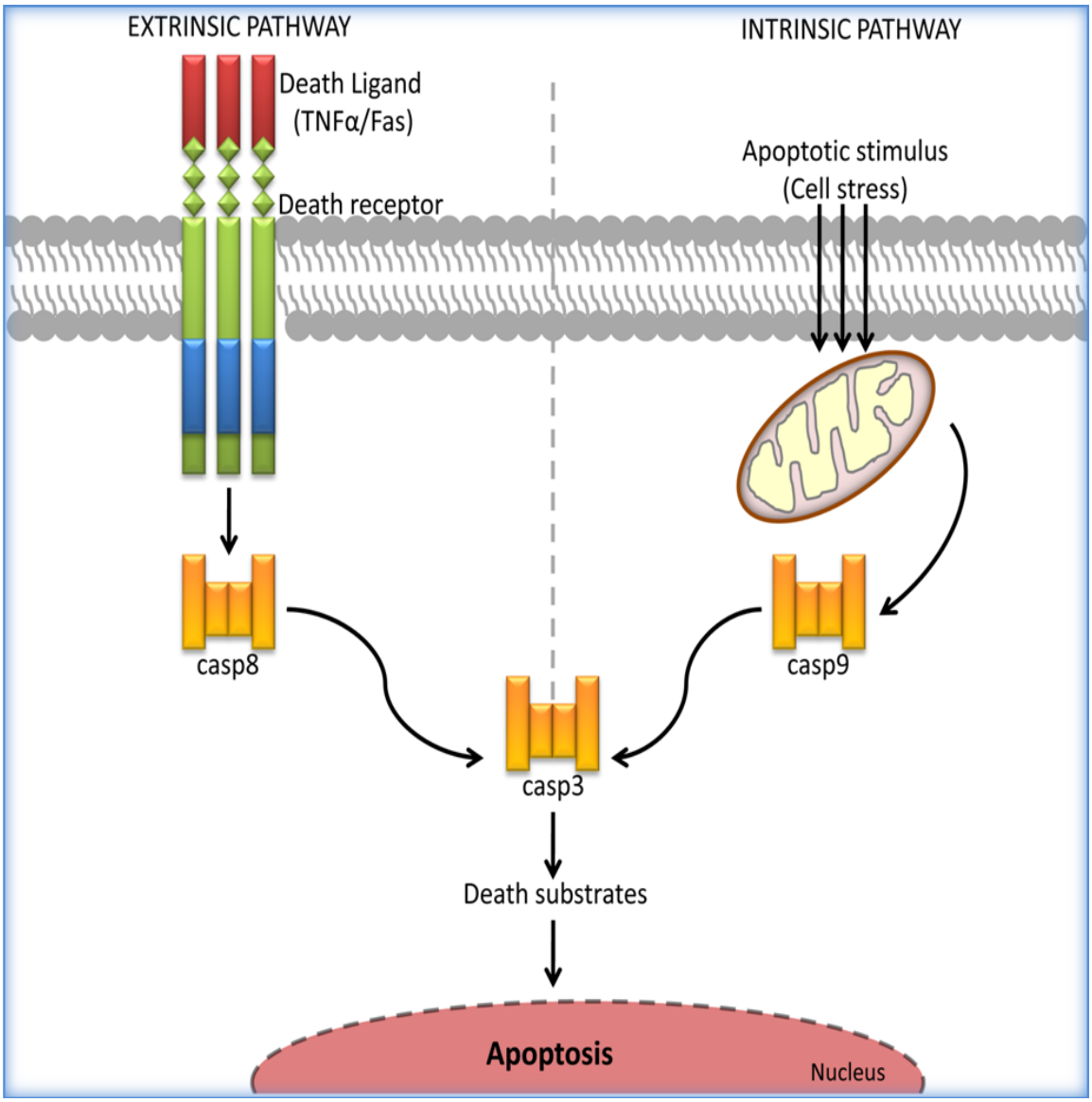



**Figure 5: Classical apoptotic pathways**

There are two main apoptotic pathways, viz, extrinsic apoptotic pathway and intrinsic apoptotic pathway. The extrinsic apoptotic pathway is triggered by death receptor engagement which involves the binding of ligand to the receptor forming a complex which helps in activation of caspase-8. Caspase-8 in turn activates the effector caspases such as caspase-3 leading to apoptosis via death substrates and other enzymatic actions. The intrinsic apoptotic pathway is triggered by cellular stresses e.g, by cytotoxic substances such as staurosporine. This pathway involves the mitochondrial outer membrane permeabilization and subsequent activation of caspase-9 and downstream effector caspase-3 resulting in the induction of apoptosis. Caspase-3 acts as a common link between extrinsic and intrinsic apoptotic pathways which finally leads to cell death.

### 1.4.1 Apoptosis in infection biology

Many pathogens are able to modulate host cell apoptosis as a mechanism of defense either by inducing this process or by inhibiting it. Some pathogens, such as, the yeast *Candida albicans* induce host cell apoptosis to evade phagocytic killing and penetrate the epithelial barrier (Ibata-Ombetta et al., 2003) while other pathogens like, *Leishmania sp.* inhibit host cell apoptosis to survive in an intracellular niche to facilitate its spreading (Ruhland et al., 2007). Pathogens have evolved several strategies to inhibit apoptosis such as prevention of cytochrome c release and protection of mitochondria, activation of cell survival pathways or prevention of caspase activation (Faherty and Maurelli, 2008). *Chlamydia* and *Neisseria* prevent cytochrome c release to inhibit apoptosis in intestinal epithelial cells (Zhong et al., 2006, Hacker et al., 2006) while as other pathogens like *Salmonella*, *Anaplasna* exploit cell survival pathways which are naturally present in the host (Knodler et al., 2005). *Shigella flexneri* inhibits apoptosis in epithelial cells by preventing the activation of caspase-3 (Clark and Maurelli, 2007). Certain pathogens like *Shigella* and *Salmonella* share dichotomous trait of inhibiting apoptosis in certain cells like epithelial cells but causes cell death in macrophages (Suzuki et al., 2005). Therefore, manipulation of host cell apoptosis is an important strategy of pathogens to establish infection of the host (Rodrigues et al., 2012; Rudel et al., 2010).

Depending on morphological forms, *A. fumigatus* has been shown to inhibit as well as induce apoptosis, for example hyphae secrete gliotoxin as secondary metabolite which induces apoptosis in various cell types including macrophages (Pardo et al., 2006, Stanzi et al., 2005). In addition, *A. fumigatus* conidia have been shown to suppress apoptosis induced in pneumocytes (Berkova et al., 2006, Femenia et al., 2009) and in macrophages (Volling et al., 2007, Volling et al., 2011). In macrophages, DHN melanin has been shown to be essential for apoptosis inhibition, however, in epithelial cells and pneumocytes, an unknown factor responsible for apoptosis inhibition has been proposed.

## 1.5 Infection models

Infection models serve as essential tools to assess host-pathogen interactions, microbial pathogenesis and virulence in order to evaluate the diagnosis, treatment, disease monitoring and therapy of fatal fungal infections. Several factors need to be considered before using an infection animal model. i) The animal model should mimic the clinical disease in patients and should be suitable for the experiment. ii) It should be reproducible and affordable. iii) It should be standardized easily.

### 1.5.1 Standard animal models

Various animal models, such as mammalian models and avian models, of *Aspergillus* diseases have been developed and used extensively from time to time (Patterson TF, 2005). Among mammalian models, mice are regarded as golden standard models for pathogenesis studies. Other mammalian models include rats, rabbits and guinea pigs (Clemons KV and Steven DA, 2005) but due to elevated costs and specialized handling skills, these models have several disadvantages. Relatively, mice are easy to handle and bear low cost and are commonly used for studying *Aspergillus* infections. In addition, mice can be genetically manipulated on the host side for investigation of disease. Various murine pulmonary and disseminated infection models have been developed (Dixon et al., 1989; Nawada et al., 1996; Sarfati et al., 2002; Sheppard et al., 2004; Stephens-Romero et al., 2005) and adapted to mimic the immune status of the host. The most commonly used are corticosteroid application or induction of neutropenia (Dagenais and Keller, 2009). Corticosteroid-based models mimic solid organ transplant recipients (Berenguer et al., 1995) which have impaired immune system and help to investigate host-pathogen interactions at various levels. A wide variety of *A. fumigatus* infections have been described in mice, including intranasal (Graybill et al., 1983), intratracheal (Williams et al., 1981), intravenous (Ford et al., 1967; Hanson et al., 1995) and inhalation (Steinbach et al., 2004; Stephens-Romero et al., 2005) infections. However, there is still need of standardization and characterization of *Aspergillus* infections in mice and other animal models.

### 1.5.2 Alternative infection models

Ethical as well as economical aspects limit the applicability of mammalian models and hence alternative infection models come into consideration. Various alternative infection models have been used which include insects such as *Drosophila melanogaster*, moth larvae such as *Galleria mellonella* and nematodes such as *Caenorhabditis elegans* (Reeves et al., 2004; Tournu et al., 2005; Ben-Ami et al., 2010a). These models bear low cost and are easy to handle with relatively reduced ethical concerns and can be used at large scale. Among these alternative models, chicken embryo infection models have also been used traditionally even before mouse strains became available. Chick embryos are used to determine the virulence of *A. fumigatus* strains (Jacobsen et al., 2010) and also used as imaging model, such as, for Positron Emission Tomography-Computed Tomography (PET/CT) (Heidrich et al., 2011). Furthermore, in the context with PET/CT and *Aspergillus* infection, chicken embryos can provide alternative model for diagnostics applications.

## 1.6 Diagnostics of *A. fumigatus* infections

Diagnosis of pulmonary *Aspergillus* infections lacks specificity and sensivity and is hampered by several issues (Subira et al., 2003) Early diagnosis is highly desirable to halt the progression of infection such as angioinvasion and dissemination which is fatal to patients. Unfortunately, there is no universal definitive test for diagnosis of *Aspergillus* infections with satisfactory results. Histological and cultural methods are often combined but require specialised expertise for species determination. Also, it takes long time to obtain the cultures and gives poor yield (Tarrand et al., 2003). In addition, histopathological features of different fungal species are indistinguishable from each other (Hayden et al., 2003). Diagnosis of infections caused by *A. fumigatus* using blood cultures often gives false positives due to contaminations as *A. fumigatus* is widespread in nature. For early diagnosis, PCR technology for detection of *Aspergillus* spp in bronchoalveolar lavage, blood and sputum are promising approaches (Bretagne et al., 2003), however, due to absence of standardized methods and tests, they are not practised routinely. Computed Tomography (CT) scan is often used for early investigations of patients suspected with invasive *Aspergillus* infections. However, there are difficulties in relying purely on CT imaging for diagnosis of *Aspergillus* infections (Kawel et al., 2011). The commonly used standard method to prove an invasive fungal

infection is histology and culturing, however due to limitations in these methods, other approaches like Galactomannan test (GM) or imaging are used to identify probable cases of these infections. The detection of galactomannan, a cell wall polysaccharide of *Aspergillus* and *Pencillium* spp which is released during fungal growth, in blood and fluids taken by bronchoalveolar lavage, is of diagnostic relevance. However, this test also has disadvantages of giving false positive results caused by various factors (Mennink Kersten et al., 2004; Klont RR et al., 2004; Mennink Kersten et al., 2005; Mennink Kersten et al., 2004). To overcome this, GM test is often combined with other methods such as Computed Tomography (CT) imaging and used for diagnosing invasive fungal infections. The fine tuning of imaging has lead to an integrated system of computed tomography and positron emission tomography (PET/CT) which is capable of providing useful diagnostic informations in a single seat from different perspectives.

### 1.7 Positron Emission Tomography (PET)/Computed Tomography (CT)

Positron Emission Tomography or PET, a nuclear medical diagnostic system, is used for imaging the functional processes in a body by producing a three-dimensional image. A PET scanner detects gamma radiations emitted by radiotracers (positron-emitting radionucleotides) that are infused into the body. Most radiotracers are chemically bound on biologically active molecules or are incorporated into compounds and get accumulated in specific regions depending on the target of the biomolecule. Tracer concentrations are acquired from three dimensional images that are constructed by computer analysis and are, thereby, correlated to their biological functions. Radiotracers are continuing to be developed for diagnosis of new processes occurring in the body with new target sites. For anatomical information, PET is often combined with radiological methods such as Computed Tomography or CT which requires no additional substances. PET combined with CT scan provides both functional as well as anatomical information during one single scan, thereby increasing sensitivity and specificity of imaging in diagnostic procedure (Fig 6) PET/CT imaging has emerged as an established method for clinical diagnostics and medical research and is one of the approaches used for diagnosis of pulmonary infections caused by pathogenic fungi, such as *A. fumigatus.*

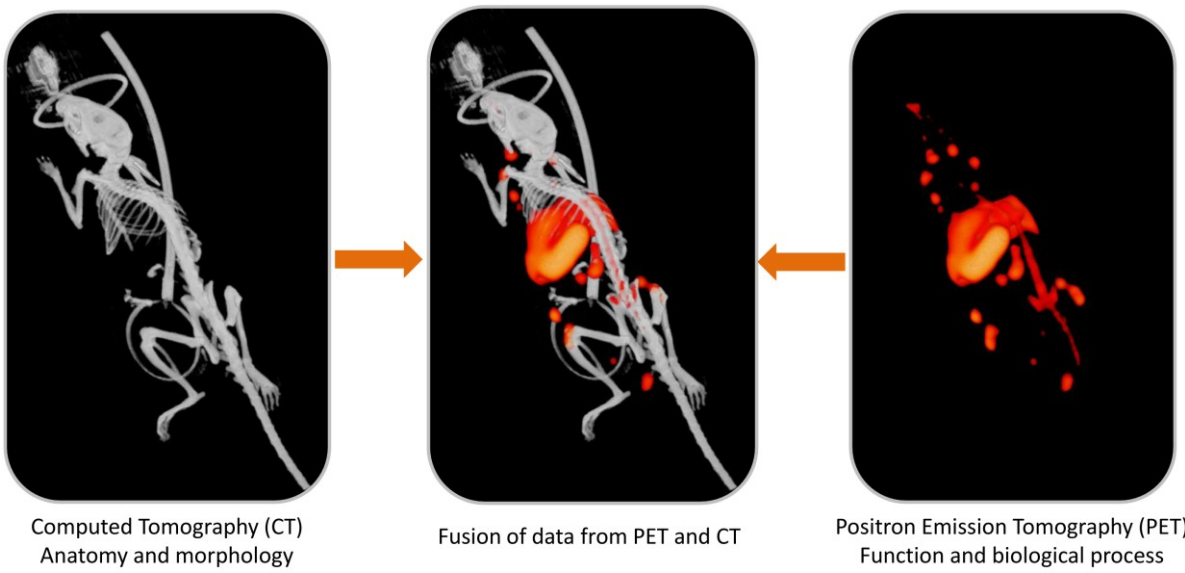



**Figure 6: Imaging by Positron Emission Tomography (PET) and Computed Tomography (CT).** PET provides the functional information of biological processes while as CT provides the anatomicalm information. The fusion of PET and CT helps to acquire the morphological and functional data almost simultaneously in one single scan.

PET employs radiotracers and several attempts have been made for development of various radiotracers specific for diagnosis of *Aspergillus* infections. Recently, Gallium-68 ($^{68}$Ga) labeled siderophore, desferritriacetylfusarinine C (TAFC), was used for imaging invasive aspergillosis in rats (Petrick et al., 2010). TAFC radiolabeled with gallium-68, a positron emitter has been evaluated as radiopharmaceutical for imaging invasive pulmonary aspergillosis. However, despite the advances in radiolabeled imaging techniques for invasive fungal infections, there is need for better tracers for fungal infection imaging and the search continues (Lupetti et al., 2011). Furthermore, in order to evaluate the radiotracers for imaging, various *in-vitro* assays are performed and to validate the *in vitro* results for different tracers, various *in vivo* models like rodents and large animals play a major role. Though these models are classified as gold standard models, however, their usage is limited due to ethical issues. Further, these models require specialized skills and facilities and bear high cost. Because of this, various alternative *in-vivo* models are taken into consideration such as moths, insects and embryonated chicken eggs which can be evaluated as imaging models for characterization and screening of novel PET tracers for fungal infections.

### 1.8 .. Siemens Inveon Small Animal microPET/CT scanner

All the imaging study was done using Siemens Inveon microPET/CT scanner. This instrument is designed for preclinal research with small animals and rodents such as mice and rats which combines PET and CT in one single device. The animals are kept on bed which can automatically move from one unit to other without changing the position of animal under experiment. The CT unit consists of X-ray source and CCD-based X-ray detector while as the PET detector is composed of 64 blocks of oxyorthosilicate scintillation crystals to detect the gamma rays. The images produced from both scanners are readily co-registered.


**Figure 7: Siemens Inveon microPET/CT instrument**
Micro PET/CT is used for preclinical experiments with small animals and combines PET and CT in one device. The subject is fixed on one platform which can be moved automatically between units. microPET/CT provides both functional and anatomical information in one single scan.

### 1.9 Siderophores as radiotracers for diagnosis of *A. fumigatus* infection.

One of the strategies of host against invading microorganisms is to withhold iron during the process of infection. To combat that, microbes possess several iron uptake systems to capture iron from the host (Schrettl M et al., 2007). Upon iron starvation, *A. fumigatus* employs two high affinity iron uptake systems: a) reductive iron assimilation (RIA) which involves reduction of ferric to ferrous iron and subsequent uptake of ferrous iron via

specialized complexes, b) siderophore-assisted iron uptake (Schrettl M et al 2004). Siderophores are low molecular mass ferric-iron specific chelators (Haas, 2003; Winkelmann G, 1993) which are upregulated during iron starvation (Schrettle M, 2010). Siderophores are actively taken up via specific iron transporters by microorganisms acquiring iron during the course of infection and activated during pathogenic growth (McDonagh et al., 2008). *A. fumigatus* produces two main siderophores, namely fusarinine C and triacetylfusarinine C (TAFC). Labeling of siderophores (TAFC) with radioisotopes such as Gallium-68 holds potential to image invasive aspergillosis uing PET/CT (Petrick et al., 2010). Besides siderophores, various attempts have been taken into account to develop radiotracers for diagnosis of invasive aspergillosis such as $^{68}$Ga-citrate (Del Val Gomiz M et al., 2000), $^{18}$F-FDG (Hot A et al., 2011), Tc-labeled polyethyleneglycol liposomes (Boerman OC et al 2000), $^{99}$Tc-interleukin-8 (Rennen HJ et al., 2004), $^{99}$Tc-fluconazole (Lupetti A et al., 2002), $^{99}$Tc-antimicrobial peptides such as ubiquicidin (Lupetti A et al., 2002, Welling MM et al., 2000), hyphal binding peptide (c(CGGRLGPFC)-NH2) labeled with $^{111}$In (Yang Z, 2009). However, the use of $^{68}$Ga-TAFC holds a promise to be a selective compound for detection of invasive pulmonary aspergillosis (Petrick et al., 2010).

### 1.10 Ligand Tracer® Technology (white)

The LigandTracer®-technology is a semi-automatically method for *in vitro* investigations of molecular interactions on cells. The system is suitable for real time measurements of radiolabelled molecules/ligands, which are able to interact with surface proteins or receptors. Ligand tracer technology gives kinetic data based on the binding of ligand in a cell covered area and offers a simple and accurate method for data acquisition of radiotracer uptake such as 2-[$^{18}$F]fluoro-2-deoxyglucose (FDG) uptake (*Techincal notes Ridgeview systems*). The system is suited for *in vitro* characteritzation of new PET tracers and is regarded as cost and time saving method to qualify the use of radiotracers on animals and humans. Ligandtracer technology is basically used for determination of affinities of radiolabeled ligands which bind to specific receptors on cell surfaces (Bjorke, H., and Andersson, K., 2006).

The core technology behind the Ligand Tracer is schematically outlined in Figure 8 below. In operation, target cells are seeded in one part of cell dish and are allowed to attach firmly to

the surface of dish. The opposite side of cell dish is used as a reference. Cell dish is placed on the holder and a small aliquot of radiotracer is added in liquid. The detector records the growing or declining response from the bound ligand representing the kinetic behavior of the interaction overtime. The differential signal (cell area minus reference area) becomes a background corrected measure of the amount of ligand attached to the cell-surface receptors.

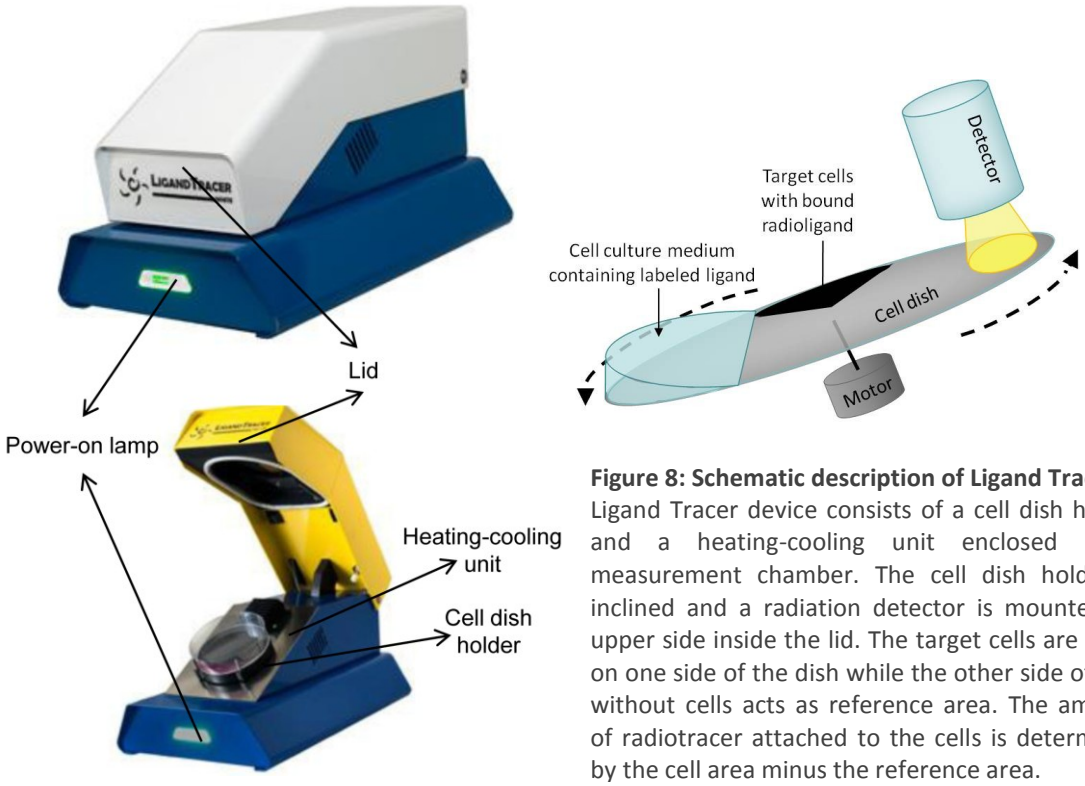



**Figure 8: Schematic description of Ligand Tracer®.** Ligand Tracer device consists of a cell dish holder and a heating-cooling unit enclosed in a measurement chamber. The cell dish holder is inclined and a radiation detector is mounted on upper side inside the lid. The target cells are sown on one side of the dish while the other side of dish without cells acts as reference area. The amount of radiotracer attached to the cells is determined by the cell area minus the reference area.

Various studies have used this *in vitro* system for determination of binding affinities of various radiolabeled ligands (Malviya et al., 2010, Ekerljung et al., 2012; Friedman et al., 2009). Though the system has found its way in binding assays in various diseases such as cancer, however, there are no reports of evaluation of Ligand Tracer technology involving infectious processes occurring from pathogenic microbes.

## 1.11 Aims of this study

Since the interaction of pathogen with its host often determines the severity of the infection, analyzing the *in vitro, in vivo and in ovo* interactions of *A. fumigatus* conidia with alveolar epithelial cells, mice and chicken eggs, respectively, provide further insights into the pathogenicity of the fungus. Various aspects of *A. fumigatus*-host interactions remain elusive and therefore, this work aimed to provide the following essential requirements:

- To analyze the role of conidial melanin on interaction of *A. fumigatus* with alveolar epithelial cells.
- To investigate the anti-apoptotic effect of *A. fumigatus* conidia on alveolar epithelial cells and the factors involved during inhibition of apoptosis.
- To evaluate mouse model for dissemination of *A. fumigatus* infection after intranasal application of conidia.
- To establish an *in vitro* protocol for evaluation of novel radiotracers for *A. fumigatus* infection using Ligand Tracer® technology.
- To establish an *in ovo* test system for evaluation of novel radiotracers for diagnosis of *A. fumigatus* infection using embryonated chicken eggs as infection model.

# 2 Materials and Methods

## 2.1 Materials

### 2.1.1 Chemicals

Unless specified, the chemicals and reagents used during this study were purchased from Carl Roth GmbH (Karlsruhe, Germany), Sigma-Aldrich (Steinheim/Seelze, Germany), VWR (Darmstadt, Germany), Merck (Darmstadt, Germany), Roche (Mannheim, Germany), Lonza (Cologne, Germany), or Bioline (Luckenwalde, Germany). The media and supplements for cell culturing were obtained from PAA Laboratories (Colbe, Germany) or Perbio/Thermo Scientific (Schwerte, Germany) or LGC Standard GmbH (Wesel, Germany).

### 2.1.2 Instruments and technical equipments

Table 1: List of instruments and technical equipments

| Instruments | Manufacturers |
|---|---|
| Analytical balance ME235S | Sartorius, Gottingen, Germany |
| Alcohol burner | Carl Roth GmbH & Co, Karlsruhe, Germany |
| Balance | Sartorius, Gottingen, Germany |
| Candling lamp | Orban Europe GmbH, Ludwigsburg, Germany |
| Centrifuge 4K15C | Sigma-Aldrich, Steinheim, Germany |
| Centrifuge Universal 32 R | Hettich Zentrifugen, Tuttlingen, Germany |
| Centrifuge 5415 R | Eppendorf, Hamburg, Germany |
| ChemiDoc XRS | Bio-Rad, Munich, Germany |
| Combi-vet® base system | Rothacher Medical GmbH, Switzerland |
| Concentrator 5301 | Eppendorf, Hamburg, Germany |
| Confocal Microscope | LSM 510, Zeiss, Jena, Germany |
| $CO_2$ Incubator IG150 | Jouan/Thermo Scientific, Schwerte, Germany |
| Digital flowmeter | Digiflow, Carbamed, Switzerland |
| Dounce tissue grinder pestle (2ml) | Sigma-Aldrich, Steinheim, Germany |
| Drilling bit 7103 Diamond | Dremel Europe, Breda, Netherlands |

| Instruments | Manufacturers |
|---|---|
| Electrophoresis Power Supply EPS 3501XL | GE Healthcare, Munich, Germany |
| Electrophoresis Power Supply EPS 600 | GE Healthcare, Munich, Germany |
| Flow cytometry | LSRII, BD Bioscience, San Jose, United States |
| Fluorescence Microscope BX-51 M | Olympus, Hamburg, Germany |
| GelVue UV-Transilluminator | Syngene, Cambridge, UK |
| Grumbach BSS300 incubator | Grumbach Brutgerate GmbH, Germany |
| Infrared lamp | Efbe Schott IR812, Germany |
| Light microscope Axiovert 25 | Carl Zeiss MicroImaging, Gottingen, Germany |
| Ligand Tracer® White | Ridgeview Instruments, Uppsala, Sweden |
| Laminar box Gera Safe | Heraeus, Hanau, Germany |
| Microwave | Siemens, Munich, Germany |
| Micro PET/CT Scanner | Siemens Medical solutions, United States |
| NanoDrop™ Nd-1000 Spectrometer | Peqlab Biotechnology, Erlangen, Germany |
| pH meter CG 840 | Schott, Mainz, Germany |
| pH meter PH 315 I Set | WTW, Weilheim, Germany |
| Spectrophotometer DU® 640 | Beckman Coulter, Krefeld, Germany |
| Speed-Vac® Plus SC210 A/SC110 | Thermo Scientific, Schwerte, Germany |
| Tank Blotting System | Bio-Rad, Munich, Germany |
| Ultrasonic processor Labsonic® M | Sartorius, Gottingen, Germany |
| Vaporizer, Dräger Isoflurane Vapor 19.3 | Drägerwerk AG & Co. KGaA, Germany |
| Vortex Genie 2™ | Carl Roth GmbH & Co, Karlsruhe, Germany |
| Water bath | GFL, Burgwedel, Germany |

### 2.1.3 Consumables

Table 2: List of plastic ware, consumables and cell culture supplements

| Consumables | Manufacturers |
|---|---|
| Blotting Paper Sheets | Munktell, Barenstein, Germany |
| Cell Culture Dishes | PAA Laboratories GmbH, Colbe, Germany |
| Cell Culture Flasks | Becton Dickinson, Heidelberg, Germany |
| Cell Scrapers (24cm/38cm) | TPP, Trasadingen, Switzerland |
| Cell Strainers (40 µm) | VWR, Darmstadt, Germany |
| Cryotubes | PAA Laboratories GmbH, Colbe, Germany |
| FACS tubes | Becton Dickinson, Heidelberg, Germany |
| Falcon Tubes (15 ml/50 ml) | Becton Dickinson, Heidelberg, Germany |
| Hybond™-PVDF Membrane | GE Healthcare, Munich, Germany |
| Microscope Slides and Coverslips | Carl Roth GmbH, Karlsruhe, Germany |
| Multiwell™ 6, 12 and 24 Well | Becton Dickinson, Heidelberg, Germany |
| Nunclon™ cell dish | Thermo Scientific, Schwerte, Germany |
| Petri dishes | Carl Roth GmbH, Karlsruhe, Germany |
| Pipette filter tip | Starlab, Ahrensburg, Germany |
| Pipette tips | Sarstedt, Numbrecht, Germany |
| Plastic cuvettes | Carl Roth GmbH, Karlsruhe, Germany |
| Serological pipettes | Becton Dickinson, Heidelberg, Germany |
| Syringe filters | Carl Roth GmbH, Karlsruhe, Germany |
| Test tubes (1.5 ml/2 ml) | Eppendorf, Hamburg, Germany |
| Thoma counting chamber | Carl Roth GmbH, Karlsruhe, Germany |
| Vacuum filtration rapid-Filtermax | Biochrom AG, Berlin, Germany |

### 2.1.4 Buffers and reagents

Table 3: List of buffer and reagents

| Name | Ingredients | Concentration |
|---|---|---|
| Ammonium persulfate (APS) solution | APS [w/v] | 10% |
| Blocking buffer (NFDM) | Tris buffered saline [v/v] | 10% |
| | Tween 20 [v/v] | 0.1% |
| | No-fat dry milk [w/v] | 5% |
| Blocking buffer (BSA) | Tris buffered saline [v/v] | 10% |
| | Tween 20 [v/v] | 0.1% |
| | BSA | 5% |
| Bradford stock solution | Ethanol (96%) | 100ml |
| | Phosphoric acid (88%) | 200ml |
| | Brilliant blue G 250 | 350mg |
| | Store at +4 in the dark | |
| Bradford working solution | $H_2O$ | 425 ml |
| | Ethanol (96%) | 15 ml |
| | Phosphoric acid (88%) | 30 ml |
| | Bradford stock solution | 30 ml |
| | Freshly used | |
| Cycloheximide (stock) | Cycloheximide in DMSO | 100 mg/ml |
| Detection buffer | Tris-HCl | 0.1 M |
| | NaCl | 0.1 M |
| | pH 9.5 | |
| DNA sample buffer (6 x) | Bromophenol blue [w/v] | 0.25 % |
| | Xylencyanol [w/v] | 0.25% |
| | Glycerol [v/v] | 30 % |
| Ethidium bromide stock solution | Ethidium bromide | 10 mg/ml |
| Laemmli sample buffer (4 x) | Glycerol [v/v] | 20 % |
| | SDS [w/v] | 4 % |
| | 2-mercaptoethanol | 1.4 M |
| | Tris pH 6.8 | 0.25 M |
| | Bromophenol blue | 50 µg/ml |
| | Store at -20 | |
| Maleic acid buffer | Maleic acid | 0.1 M |
| | NaCl | 0.15 M |
| | Adjust pH to 7.5 (NaOH) | |

| Name | Ingredients | Concentration |
|---|---|---|
| PAA separation gel (10%) | PAA stock solution (30%) | 1.65 ml |
| | $H_2O$ | 1.8 ml |
| | Solution B | 1.25 ml |
| | TEMED | 5 µl |
| | APS (10% [w/v]) | 30 µl |
| PAA Separation gel (12.5%) | PAA stock solution (30%) | 1.7 ml |
| | $H_2O$ | 1.3 ml |
| | Solution B | 1 ml |
| | TEMED | 4 µl |
| | APS (10% [w/v]) | 24 µl |
| PAA Stacking gel (3.75%) | PAA stock solution (30%) | 0.3 ml |
| | $H_2O$ | 1.5 ml |
| | Solution C | 0.6 ml |
| | TEMED | 2.5 µl |
| | APS (10% [w/v]) | 12.5 µl |
| Panning buffer | NaCl | 150 mM |
| | Tris | 50 mM |
| | $CaCl_2$ | 1 mM |
| | pH 7.4 | |
| PBS pH 7.4 | NaCl | 8 g/l |
| | KCl | 0.2 g/l |
| | $Na_2HPO_4$ | 1.15 g/l |
| | $KH_2PO_4$ | 0.2 g/l |
| | Adjust pH to 7.4 (HCl or NaOH) | |
| Phosphate citrate (PC) buffer | $Na_2HPO_4$ (0.2 M) | 192 parts |
| | Citric acid (0.1 M) | 8 parts |
| | pH 7.8 | |
| PI-Rnase A solution | Propidium iodide | 50 µg/ml |
| | Sodium citrate [w/v] | 0.1 % |
| | Triton® X 100 [w/v] | 0.1% |
| | Rnase A | 100 µg/ml |
| Propidium iodide (stock) | Propidium iodide in sodium citrate buffer | 2 mg/ml |
| Pyrithiamine solution | Pyrithiamine | 1mg/ml |
| SDS PAGE buffer (10 x) | Tris | 30.2 g/l |
| | Glycine | 188 g/l |
| | SDS | 10 g/l |
| Solution B | Tris | 1.5 M |
| | SDS [w/v] | 0.4 % |
| | pH 8.9 | |

| Name | Ingredients | Concentration |
|---|---|---|
| Solution C | Tris<br>SDS [w/v]<br>Ph 6.8 | 0.5 M<br>0.4% |
| Staurosporine | Staurosporine in DMSO | 1.5 M |
| Stripping buffer | 2-mercaptoethanol<br>Tris pH 6.8<br>SDS [w/v] | 100 mM<br>62.5 mM<br>2% |
| TAE buffer (10 x) | Tris pH 8<br>EDTA<br>Glacial acetic acid<br>pH 8.5 | 0.4 M<br>0.01 M<br>0.2 M |
| TBS buffer (10x) | Tris pH 8.0<br>NaCl<br>KCl | 500 mM<br>1.37 M<br>27 mM |
| TBS buffer (1 x)+ Tween 20 (TBST) | TBS buffer (10 x)[w/v]<br>Tween 20 [v/v] | 10 %<br>0.1 % |
| TNF-α<br>Cycloheximide | In PBS<br>In DMSO | 80 ng/ml<br>2 µg |
| Transfer buffer (1 x) | SDS PAGE buffer (10 x)<br>EtOH [v/v] | 10 %<br>20 % |
| Tris-HCl 1 M (pH 6.8?7.5/8.0) | Tris<br>Adjust pH with HCl | 121.14 g/l |

### 2.1.5 Enzymes and Inhibitors

Table 4: List of enzymes and inhibitors

| Enzymes/Inhibitors | Manufacturers |
|---|---|
| Cycloheximide | Sigma-Aldrich (Steinheim, Germany) |
| Halt™ Protease & Phosphatase Cocktail | Thermo Scientific (Schwerte, Germany) |
| Proteinase K | Boehringer (Ingelheim, Germany) |
| Recombinant Murine TNF-α | Sigma-Aldrich (Steinheim, Germany) |
| Ribonuclease A | Carl Roth GmbH (Karlsruhe, Germany) |
| Staurosporine | Sigma-Aldrich (Steinheim, Germany) |

### 2.1.6 Primary and secondary Antibodies

Table 5: Primary antibodies used for Western blot analysis

| Antibody | Host | Manufacturers | Dilution |
|---|---|---|---|
| β-actin | Mouse | Sigma-Aldrich, Steinheim, Germany (#A54316) | 1:3500 in TBST + 5% NFDM |
| Caspase-3 | Rabbit | Cell Signaling/NEB, Frankfurt, Germany (#9662) | 1:1000 in TBST + 5% NFDM |
| Cleaved caspase-3 (Asp 175) | Rabbit | Cell Signaling/NEB, Frankfurt, Germany (#9761) | 1:1000 in TBST + 5% NFDM |

Table 6: Secondary antibodies used for Western blot analysis

| Antibodies | Host | Manufacturers | Dilution |
|---|---|---|---|
| HRP-conjugated anti-rabbit IgG | Goat | Dianova, Hamburg, Germany (#111-035-003) | 1:5000 in TBST + 5% NFDM |
| HRP-conjugated anti-mouse IgG | Sheep | Sima-Aldrich, Steinheim, Germany (#A 5316) | 1:5000 in TBST + 5% NFDM |

### 2.1.7 Markers

Table 7: DNA and protein markers

| Marker | Company |
|---|---|
| GeneRuler™ 100 bp DNA Ladder Plus | Fermentas (St. Leon-Rot, Germany) |
| MassRuler™ DNA Ladder, Low Range | Fermentas (St. Leon-Rot, Germany) |
| PAGE Ruler™ Plus Prestained Protein Ladder | Fermentas (St. Leon-Rot, Germany) |

### 2.1.8 Kits

Table 8: List of commercially available kits

| Kits | Manufacturers |
|---|---|
| VenorGeM Mycoplasma Detection Kit (MP00025) | Biochrom AG (Berlin, Germany) |
| Qiagen DNA extraction kit | Qiagen GmbH (Germany) |
| Phusion High-Fidelity DNA Polymerase | Finzymes, Thermo-scientific (Germany) |

### 2.1.9 *Aspergillus* culture media

Table 9 provides the list of media and trace elements used for the cultivation of *Aspergillus* strains. To prepare solid medium, 20 g/l difco agar was added after adjusting the pH to 6.3-6.5. The supplements and Hutner's trace elements were sterile filtred and kept at 4 $^{o}$C for storage whereas, the media was autoclaved. To prepare trace elements, the solution was heated to boiling, cooled to about 60 $^{o}$C and finally adjusting the pH to 6.5-6.8. The solution turns deep purple after standing for several days.

Table 9: Media and trace elements for cultivation of *Aspergillus* strains

| Culture medium | Ingredients | Concentration |
|---|---|---|
| Aspergillus minimal medium (Brakhage and Van den Brulle, 1995; Pontecorvo et al., 1953) | $NaNO_3$ | 6 g/l |
| | $KH_2PO_4$ | 1.52 g/l |
| | KCl | 0.52 g/l |
| | Adjust pH 6.3 – 6.5 (NaOH/KOH) | |
| | Autoclave and then add, | |
| | $MgSO_4$ (20% [w/v]) | 2.5 ml/l |
| | Glucose (20% [w/v]) | 50 ml/l |
| | Hutner's trace elements | 1 ml/l |
| Yeast malt extract medium | Malt extract | 20 g/l |
| | Yeast extract | 1 g/l |
| | Glucose | 10 g/l |
| | $NH_4Cl$ | 0.25 g/l |
| | $K_2HPO4$ | 0.25 g/l |
| Hutner's trace elements (1000 x) | $ZnSO_4$. 7H2O | 2.2 g/100 ml |
| | $H_3BO_3$ | 1.1 g/100 ml |
| | $MnCl_2$. 4H2O | 0.5 g/100ml |
| | $FeSO_4$. 7H2O | 0.5 g/100ml |
| | $CoCl_2$. 6 H2O | 0.16 g/100ml |
| | $CuSO_4$. 5H2O | 0.16 g/100ml |
| | $(HH_4)6Mo_7O_{24}$. 4H2O | 0.11 g/100ml |
| | $Na_4EDTA$. 4H2O | 6.0 g/100ml |

### 2.1.10 *Aspergillus* strains

Table 10: *Aspergillus* strains used in this study

| Strain | Genotype | Source of reference |
|---|---|---|
| ATCC 46645 | Wild type | American Type Culture Collection |
| CEA10 (CBS144.89) | Wild type | American Type Culture Collection |
| *pksP* mutant | Non melanised | Langfelder *et al*., 1998 |
| CEA17 | Uracil auxotroph | D'Enfart *et al.,*1996 |
| CEA17pksP | Non melanised uracil auxotroph | This study |
| *hapX* mutant | ATCC 46645 *ΔhapX* | Schrettl *et al*., 2010 |

### 2.1.11 Cell lines

The human cell line A549 (ATCC-CCL-185$^{TM}$) and murine cell line MH-S (CRL-2019) were obtained from American Type Culture Collection (ATCC). A549 is an immortalized alveolar type II pulmonary epithelial cell line which was obtained from lung carcinomatous tissue of 58 year old Caucasian male (Giard et al., 1972). Cells have a doubling time of about 22 h and bear the typical morphological features of type II pneumocytes. MH-S is alveolar macrophage cell line which was obtained by bronchoalveolar lavage from Balb/cJ mice (Mbawuike and Herscowitz, 1989). The MH-S cells have a doubling time of about 48 h with typical macrophage morphology and functions.

### 2.1.12 Radiotracer

Gallium-68 ($^{68}$Ga) labeled desferri-triacetylfusarinine C (TAFC), previously used for imaging *A. fumigatus* infection in rats (Petrick et al., 2010), was used to trace the infection in embryonated chicken eggs. The radiotracer was prepared by lab chemist (Peter Gebhart) as described previously (Gebhardt et al., 2010) and the radioactivity of $^{68}$Ga-TAFC was monitored before application of tracer to target. The tracer was diluted in cold PBS.

### 2.1.13 ..Imaging system and analysis

All PET/CT scans were performed with Siemens Inveon Small Animal microPET/CT scanner (Siemens Medical Solutions, Siemens Healthcare Molecular, USA) by imaging specialists (Thomas Opfermann and Alexander Heidrich). The final images from CT and PET were fused

together and the regions of interest (ROI) were selected. The mean activity concentration at different ROIs was calculated using Siemens Inveon Research Workplace Software (IRW, version 3.0, Siemens Medical Solutions, Siemens Healthcare Molecular Imaging, USA).

### 2.1.14 Software and online data bases

Table 11: Software and data bases used in this study

| Softwares | Manufacturers |
|---|---|
| Adobe Photoshop CS3 | Adobe Systems, Unterschleissheim, Germany |
| Cell D Life Science documentation software | Olympus, Hamburg, Germany |
| GeneGenius Gel Documentation and Analysis System | Syngene, Cambridge, United Kingdom |
| Microsoft Office 2007 | Microsoft Deutschland GmbH, bad Homburg, Germany. |
| Quantity One 4.6.1 | Bio-Rad, Munich, Germany |
| Tracer Drawer 1.3 | Ridgeview Instruments, Uppsula, Sweden |
| Ligand Tracer 1.0.2 | Ridgeview Instruments, Uppsula, Germany |

## 2.2 Methods

### 2.2.1 *Aspergillus* strains and culture conditions

The list of *Aspergillus* strains used in this study is provided in Table 10. In addition to *A. fumigatus* wild-type strains CEA10 (CBS144.89) and ATCC 46645 (American Type Culture Collection, Manassas, VA), two uracil auxotrophic *A. fumigatus* strains were used: CEA17 (D'Enfert et al., 1996) which is a derivative of CEA10 characterized by a point mutation in the *pyrG* gene and CEA17pksP, which is a non-melanised mutant obtained by partial deletion of the *pksP* gene in CEA17. *A. fumigatus* was cultivated on *Aspergillus* minimal medium (AMM) or malt extract medium and uracil auxotrophs were supplemented with 10 mM uracil and 5 mM uridine as described previously (Weidner et al., 1998).

### 2.2.2 Preparation of conidial spore suspension

The dormant conidia grown on agar plates were harvested from 5 to 7 days old cultures. Uracil auxotrophic conidia were harvested on day 7 unless and otherwise stated. To prepare conidial suspensions, plates were rinsed with 10 – 15 ml of sterile phosphate buffered saline (PBS) supplemented with 0.1% (v/v) Tween 20 (PBST) or 0.9% (w/v) sodium chloride (NaCl) supplemented with 0.1% (v/v) Tween 20. The conidia were carefully suspended with a rubber policeman and filtered through cell strainer with pore size of 40 µm (BD Bioscience, Heidelberg, Germany). The concentration of spore suspension was calculated by microscopical enumeration using haemocytometer (Thoma chamber) according to the classical procedure of spore counting. Resting spores were fixed by washing the spores with PBS and suspending in 2.5% (v/v) *p*-formaldehyde at 4° C overnight. The fixed spores were neutralized with 0.1 M ammonium chloride ($NH_4Cl$) and resuspended in PBS prior to addition to cells.

### 2.2.3 Fluorescein-5-isothiocyanate (FITC)-labeling of conidia

Prior to infection for microscopical studies, *A. fumigatus* conidia were stained with fluorescein-5-isothiocyanate (FITC) (Sigma-Aldrich, Steinheim, Germany) as described before (Sturtevant and Latge, 1992). Briefly, resting conidia were harvested and suspended in 0.1 M carbonate buffer (pH 9.3) with 0.1 mg/ml FITC. The suspension was incubated for 1 h at 37

°C in dark in a rotatory incubator. Conidia labeled with FITC were washed three times in PBS supplemented with 0.05% Tween 20 (PBST) by centrifugation at 5000 x g for 10 min each time. After washing, the conidia were resuspended in PBST and counted. FITC-labeled conidia were directly used for infection of cells with storage period of maximum 24 h at 4 °C.

### 2.2.4 Preparation of melanin ghosts

Melanin ghosts from *A. fumigatus* wild-type conidia were prepared as described previously (Youngchim et al. 2004; Volling et al., 2011). The freshly harvested dormant conidia (2 x $10^9$) were washed three times with PBS and the conidial cell wall was digested overnight at 30 °C with 10 mg/ml Glucanex® (Novozymes, Bad Kreuunach, Germany) in 0.1 M sodium citrate and 1 M sorbitol (pH 5.5). The protoplasts were washed three times with PBS followed by overnight incubation at room temperature in 4 M guanidine thiocyanate solution (Fluka Biochemika/Sigma-Aldrich, Steinheim, Germany). After washing three times with PBS, the generated particles were treated with proteinase K solution (1 mg/ml proteinase K from *Triterachium album* (Boehringer Ingelheim, Germany)), 1 mM calcium chloride ($CaCl_2$), 10 mM Tris, and 0.5 % (w/v) SDS at 37 °C overnight to remove the residual proteins. The pellet was again washed three times with PBS before boiling for 90 min in 6 M hydrochloric acid to obtain pure melanin. The generated melanin particles (melanin ghosts) were collected by filtration through 0.45 µm microporous membrane Ultra-MC Centrifugal Filter Units (Milipore, Schwalbach, Germany) and washed three times with PBS. The final step was done by lyophilization of melanin ghosts and resuspension in PBST. The melanin ghost suspension was stored at 4 °C until used. In addition to melanin ghosts, synthetic DOPA-melanin (Sigma-Aldrich, Steinheim, Germany) and natural melanin from *Sepia officinalis* (Sigma-Aldrich, Steinheim, Germany) were also used in the study.

### 2.2.5 Deletion of the *pksP* gene

Isolation and manipulation of genomic *A. fumigatus* DNA and Southern blot analysis were carried out as previously described (Grosse et al., 2008). Briefly, for isolation of genomic DNA, *A. fumigatus* was grown in liquid AMM for 24 h. Chromosomal DNA of *A. fumigatus* was prepared using Master Pre Yeast DNA Purification Kit (Epicenter Biotechnologies, USA). For Southern blot analysis, DNA fragments were separated on an agarose gel and blotted

onto Hybond N+ nylon membranes (GE Healthcare Bio-Sciences, Germany). Labeling of DNA probes, hybridization and detection of DNA-DNA hybrids were performed as described before (Grosse et al., 2008). Partial deletion of the *pksP* gene was achieved by employing a PCR based strategy. Fragments of the *pksP* gene were amplified by PCR using primer pairs pksP1707_for and pksP2744ptrA_rev and pksP3723ptrA_for and pksP4745_rev, respectively (Table 12). By this reaction, overlapping ends to the pyrithiamine resistance cassette were introduced at the 3'-end of the upstream region and at the 5'-end of the downstream region of the *pksP* gene. The *ptrA* resistance cassette was amplified from plasmid pSK275 (Szewczyk and Krappmann, 2010) with primers ptrA-for and ptrA-rev. The final deletion construct was generated by a three fragment PCR employing primers pksP1707_for and pksP4745_rev. All PCR reactions were performed with Phusion High-Fidelity DNA Polymerase (Finnzymes) according to the manufacturer's recommendations. The resulting 5 kb PCR product was used for transformation of *A. fumigatus* CEA17 protoplasts. Pyrithiamine (1 mg/ml, Sigma-Aldrich) resistant transformants that produced white conidia were analysed for partial deletion of *pksP* by Southern blot analysis. One positive transformant, designated CEA17pksP, was chosen for further analysis.

Table 12: Oligonucleotides used in this study

| Oligonucleotide | Sequence 5´-3´ |
|---|---|
| pksP1707_for | CATCAGTGGTGGTGTAAACC |
| pksP4745_rev | GCGATAATGTCATCCCCTTC |
| pksP2744ptrA_rev | GGCCTGAGTGGCCATCGAATTCGTGAAGACGAACCCAGTCTTG |
| pksP3723ptrA_for | GAGGCCATCTAGGCCATCAAGC CTTCAGCTCGGCTCACGAAG |
| ptrA_for | GAA TTC GAT GGC CAC TCA GGC C |
| ptrA_rev | GCT TGA TGG CCT AGA TGG CCT C |

### 2.2.6 Cell cultures

The human type II pneumocyte cell line A549 was maintained in F-12K medium (LGC Standards GmbH, Germany) supplemented with 10 % (v/v) heat inactivated fetal bovine serum (FBS) and 0.5 µg/ml gentamicin (PAA Laboratories GmbH, Cölbe, Germany). Cells were grown until 60-70 % confluent and detached by using 0.25 % (w/v) trypsin-0.5 mM EDTA

solution for sub-culturing. Murine alveolar macrophage cell line MH-S was cultured in RPMI 1640 medium supplemented with 10% (v/v) heat inactivated FBS (Perbio/Thermo Scientific, Schwerte, Germany) and 20 mg/ml gentamicin (PAA Laboratories GmbH, Cölbe, Germany). All incubations were carried out at 37 °C in a humidified incubator with 5% (v/v) $CO_2$. Cells were frequently checked for mycoplasma contamination using VenorGeM Mycoplasma Detection Kit (Biochrom AG, Berlin, Germany) as per the manufacturer`s instructions.

### 2.2.7 Freezing and thawing of cell cultures

A549 cells, for cryoconservative stocks, were grown in T-175 flasks and detached, when 80 – 90 % confluent, using trypsin-EDTA solution as described above. The cell suspension was transferred to 15 ml Falcon tube and collected by centrifugation for 5 min at 300 x g. The cell pellet was suspended in 4 ml of freezing medium composed of FBS and 10 % DMSO (v/v). 1 ml of suspension was added to four sterile cryotubes and kept on ice. This was followed by transferring the tubes to a freezing rack and storing at -20 °C for 2 h. Afterwards, the tubes were transferred to –80 °C and kept overnight. For long term storage, the cells were kept in liquid nitrogen. For thawing the cells, frozen vials were removed from storage and kept directly on ice. Gradually, cells were thawed by resuspending in pre-heated culture medium and transferring the thawed cells to culture flask containing pre-heated medium. The process was carried untill all cells were defrosted and suspended. The cells were incubated at 37 °C with humidified atmosphere of 5 % (v/v) $CO_2$ overnight and the medium was replaced next day with fresh medium without DMSO. The viability of cells was examined using trypan blue (0.4% w/v).

### 2.2.8 Phagocytosis assay

A549 cells were seeded on glass cover slips in 24-well plates (5 x $10^5$ cells per well) in triplicates and allowed to grow adherently for 16 h at 37 °C in humidified atmosphere of 5 % (v/v) $CO_2$. The cells were washed with pre-warmed F-12K medium without supplements and challenged with fluorescein-5-isothiocyanate (FITC)- labeled conidia in F-12K medium at multiplicity of infection (MOI) of 5. For co-cultures of *A. fumigatus* wild-type and *pksP* conidia with A549 cells, wild type conidia were labeled with FITC while as *pksP* conidia were incubated without any stain. To synchronize phagocytosis, cells with condia were incubated

at 4 °C for 30 min. The phagocytosis was initiated by transferring the cells to 37 °C in 5 % (v/v) $CO_2$). After incubation for 6, 12 and 24 h at 37 °C in 5 % (v/v) $CO_2$, unbound conidia were washed with pre-warmed supplement free F-12K medium and phagocytosis was stopped by adding ice-cold PBS to the wells. Extracellular conidia were labelled with 0.25 mg/ml calcofluor white/PBS (Sigma-Aldrich Germany) for 30 min at 4 °C in the dark followed by three times gentle washing with PBS. As mammalian cells are impermeable for calcoflour white, only phagocytosed conidia retained their FITC signal, whereas non-phagocytosed conidia displayed the calcofluor white fluorescence. Fixation of cells was carried out with 4 % (v/v) Rotifix (Carl Roth, Germany) for 15 min at room temperature and washing three times with PBS. The coverslips were mounted onto glass slides for microscopy and examined using a Zeiss LSM 5 Live confocal laser scanning microscope. Two random fields containing at least 100 cells per field of three independent experiments were used for determination the percentage of phagocytosis, which was calculated as the average number of conidia that were phagocytosed per phagocyte of a total of 100 phagocytes.

### 2.2.9 Nystatin survival assay

$5x10^5$ cells were seeded in 12 well plates in triplicates one day prior to infection with *A. fumigatus* and incubated at 37 °C with humidified atmosphere of 5% (v/v) $CO_2$ as described above. The cells were washed with pre-warmed medium as described in section 2.2.8 for the phagocytosis assay and infected with conidial suspension at an MOI of 5. After co-incubation for 6, 12 and 24 h, the cells were washed three times with PBST and incubated with 100 mg/ml nystatin (Sigma-Aldrich, Germany) in F-12K medium for 3 h. This concentration of nystatin was shown to kill $10^6$ conidia and did not affect the viability of cells (Wasylnka and Moore, 2002). After 3 h, the cells were lysed with 0.5 % (v/v) Triton X-100 for 10 min and released conidia were plated on yeast malt agar plates. The colonies were counted after 24 h to determine the survival rate of internalized conidia which was calculated as the number of colonies recovered from nystatin-treated wells divided by the number of colonies recovered from nystatin untreated wells.

### 2.2.10 Induction and inhibition of apoptosis

For induction of apoptosis in A549 lung epithelial cells, 80 ng/ml tumor necrosis factor-α (TNF-α; Sigma-Aldrich, Steinheim, Germany) was simultaneously applied with 2 µg mRNA translation inhibitor cycloheximide (CHX) (Sigma-Aldrich, Steinheim, Germany) for induction of the extrinsic, receptor-mediated apoptosis pathway (Berkova et al., 2006; Volling et al., 2007), which suppress survival signals induced by engagement of TNF-α receptors (Higuchi et al., 1995). Treatment of A549 cells with TNF-α alone did not induce apoptosis when analyzed 6 h after administration. For investigation of intrinsic, mitochondrial-mediated pathway, the kinase inhibitor staurosporine (STS) was used for inducing apoptosis (Banga et al., 2007; Harkin et al., 1998) at final concentration of 1.5 µm. The general caspase inhibitor Z-VAD-FMK (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone, Sigma-Aldrich, Germany) was used to check the involvement of the caspase cascade in the apoptotic process. 0.25 mM Z-VAD-FMK was added to the cultures 1 h before induction of apoptosis. As controls, the cultures with *A. fumigatus* conidia were compared to the cultures either with the inducer or solely with the medium.

### 2.2.11 Flow cytometry analysis

To analyze the extent of DNA fragmentaion which defines the late stage of apoptosis, the hypodiploid or subG1 DNA content was measured by propidium iodide staining using flow cytometry analysis. Conidia were labelled with FITC (Sigma-Aldrich) as described previously (Sturtevant and Latge, 1992) in section 2.2.3. FITC-labelled conidia were co-incubated with A549 cells at MOI of 0.5 for 6, 8, 10, 12 and 24 h. To induce apoptosis, A549 cells were treated with the apoptotic stimuli TNF-α/CHX or STS for 6 h. Conidia were co-incubated with A549 cells in F-12K medium without supplements at 37 °C and 5 % (v/v) $CO_2$. Since apoptotic cells detach from the surface, the supernatant was collected and adherent cells were combined with the supernatant after gentle scrapping using 500 µl PBS. The washed pellet was prefixed in 70 % (v/v) ethanol and stored at -20 °C. The samples were centrifuged at 800 g for 5 min and the cell pellet was incubated in 40 µl phosphate-citric acid buffer (0.2 M $Na_2HPO_4$ and 0.1 M citric acid in a 24:1 ratio) at pH 7.8. DNA was selectively extracted from the cells for 30 min at room temperature. The remaining cells with fractional DNA content were pelleted and used for flow cytometry (Gong et al., 1994; Volling et al., 2007).

### 2.2.12 SDS- Page and Western blot analysis

For analysing the presence of both pro-caspase-3 and active caspase-3, A549 cells were lysed using NP-40 lysis buffer (10 mM NaCl, 1 % (v/v) NP-40, 50 mM Tris/HCl, pH 8)) and Halt Protease & Phosphatase Inhibitor Single Use Cocktail (Thermo Scientific), followed by mild sonification on ice. Cellular debri was precipitated at 10000 x g by centrifugation for 5 min. Protein concentration was assessed by Bradford assay and protein lysates were stored at -20 °C. 25-30 µg of the protein extract were boiled for 5 min in 4 x Laemmli buffer. The samples were loaded on an SDS-PAGE for separation of proteins (Laemmli, 1970) and transferred onto a 0.45 µm Immuno-Blot polyvinylidene difluoride (PVDF) membrane (GE-Healthcare, Munich, Germany) in a blottinmg chamber (Bio-Rad, Munich, Germany) at 200 mA per gel or 300 mA per two gels for 90 min. The membranes were washed thrice with TBST (150 mM NaCl, 10 mM Tris x HCl, pH 7.5, 0.1% (v/v) Tween 20) for 5 min and blocked with 5 % (w/v) non-fat dry milk for 1 h. Polyclonal rabbit anti caspase-3 antibodies (1:1000; Cell Signaling) were used for detection. After washing with TBST three times, the primary antibody was applied overnight at 4 °C on rotating platform in TBST supplemented with 5 % (w/v) non-fat dry milk. The membrane was washed three times with TBST for 10 min followed by addition of secondary antibody for 1 to 2 h at room temperature in TBST supplemented with non-fat dry milk. The membrane was again washed with TBST for 10 min three times and the protein-antibody complexes were visualized using an enhanced chemiluminescence-based detection system (GE-Healthcare, Munich, Germany). The calculation of the protein band intensity was performed with Quantity one (Bio-Rad, Munich, Germany) according to the manufacturer's instructions. For measuring caspase-3 activity, the relative caspase-3 level in each sample was calculated by dividing the values of band intensities of cleaved caspase-3 by the value of band intensity corresponding to pro-caspase-3. β-actin antibody (1:1000; Sigma-Aldrich, Germany) was used as loading control to compare the amount of protein loaded in each sample.

### 2.2.13. Stripping of Western blots

For reprobing a Western blot with several antibodies, PVDF membranes were incubated at room temperature for 30 to 45 min in stripping buffer followed by extensive washing in TBS with final washing step in TBST for 10 min. The membrane was blocked as described in

section 2.2.12 above and reprobed with new primary antibody.The above procedure was followed with secondary antibody and detection.

### 2.2.14. Murine infection models

Female CD-1 mice (Charles River Laboratories, Germany), 6 - 8 weeks old weighing about 18 - 20 g, were used in all studies. Mice were kept in ventilated cages with a maximum of 4 mice per cage and taken care in accordance to the principles outlined in the *European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes* (http://conventions.coe.int/Treaty/en/Treaties/Html/123.htm). Animal experiments were approved by Federal State authority (Thuringer Landesamt fur Verbraucherschutz) and ethics committee (permit no. 03-002/09; 03-012/10) and were in accordance with the German animal welfare act. Animals were monitored at least twice daily and humanely sacrificed via intraperitoneal administration of an overdose of anesthetic (ketamine/xylazine (Inresa Arzneimittel, Freiburg, Germany)) in combination with blood withdrawal. In deep anesthesia (anesthesis stage 4: cessation of spontaneous respiration), a maximum blood sample was taken by cardiac puncture. Animals which were found to have disturbed general condition, which suggests a very severe course of infection, were euthanized immediately. Animals were sacrificed on days 1, 3, 5, 10 and 15 after infection. Organ samples for analyses of fungal burden were collected during post mortem analysis.

### 2.2.15. Intranasal infection

For infection, the mice were anesthetized using a mixture of fentanyl (0.05 mg/kg, Janssen-Cilag, Neuss, Germany), midazolam (5 mg/kg, Roche, Mannheim, Germany) and medetomidin (0.5 mg/kg, Fort Dodge Veterinar GmbH, Wurselen, Germany) by intraperitoneal injection. To maintain the body temperature, the anesthetized animals were kept on heat pads in dorsal position with head lifted up. The freshly harvested spore suspension (20 µl in PBS) was applied in drops on the nostrils and inhaled by the mice. The infection dose depends on the part of the experiment and is mentioned in the sections 2.2.16 and 2.2.17. The day of infection was designated as day 0.

### 2.2.16. Corticosteroid mouse model

25 mg of cortisone acetate was injected intraperitoneally per mouse at day -3 and day 0. Mice were challenged intranasally on day 0 with $1 \times 10^4$ conidia in 20 µl PBS.

### 2.2.17. Immunocompetent mice

To investigate infections in immunocompetent mice, untreated female specific pathogen-free CD-1 mice were challenged intranasally with $1 \times 10^6$ conidia in 20 µl PBS.

### 2.2.18 Histopathologocal analysis

For histological analysis, the organs were fixed in histofix (Carl Roth, Germany) embedded in paraffin and sectioned. 5 µm sections were stained with hematoxylin eosin (HE) or PAS reaction according to standard protocols.

### 2.2.19 Embryonated chicken egg model

Fertilized *Gallus gallus domesticus* (White Leghorn chicken) eggs were obtained from Geflügel GmbH Borna (Germany). All experiments were conducted as per the German animal protection law. According to this law, there is no specific approval required to work with embryonated chicken eggs before the time of hatching. Due to this reason, all experiments were terminated on or before developemental day 18 by chilling the eggs on ice for 30-60 min. The eggs were incubated at 37.6 °C and a relative humidity of 60% in a forced-air egg incubator (Grumbach BSS300 MP GTFS incubator; Grumbach Brutgeräte GmbH, Germany) to avoid dehydration. After the fourth day of incubation until they were infected, the eggs were turned automatically four times a day by 360 degree in order to avoid sticking of embryo with the shell membrane.

### 2.2.20 Infection of embryonated chicken eggs via chorio-allantoic membrane (CAM)

Infection of eggs was performed via chorioallantoic membrane (CAM) as described (Jacobsen et al. 2010) on developmental day 10. Briefly, on the day of infection, the egg shell was disinfected and small area was perforated with a dentist drill and hook. An artificial air chamber was created by applying pressure with the help of peleusball. 100 ul of fungal suspension containing $10^4$ spores were applied directly on the CAM. The viability of chicken

eggs was checked daily by candling. Ten eggs per group were infected with *A. fumigatus* spores ($10^4$ spores/egg), from which three eggs were randomly selected for micro-PET imaging three days post infection (p.i.). The control group (n=6) was inoculated with PBS and three eggs were randomly selected for imaging.

### 2.2.21… Determination of fungal burden by colony forming units and quantitative real time PCR.

After aseptically removing the organs (in mice models) and visible fungal spots of CAM (in chick embryo model), the organs and the CAM were weighed and primary homogenates were prepared in sterile saline by direct pressure. For colony forming units, primary homogenates were serially diluted, spread on malt agar plates and incubated at 37 °C for 24 to 48 h before *A. fumigatus* colonies were counted.

For DNA isolation, primary homogenates were transferred to sterile micro centrifuge tubes and 0.5 mm diameter glass beads were added. The samples were mechanically disrupted by beat beating at 3200 rpm with three bursts of 30 s and incubating on ice between bursts. This secondary homogenate was centrifuged and DNA was isolated using Qiagen DNA mini kit as per manufacturer's protocol. The quality and quantity of isolated DNA was determined by using nanodrop spectrophotometer (Peqlab Biotechnology, Germany). The concentration of DNA was adjusted to 50 ng/µl and previously described primers and probes for 18s rRNA were used for detection of *A. fumigatus* in samples (Bowman et al., 2001). DNA samples were analyzed in triplicates to determine the fungal DNA.

Table 14: Primers and probes used:

| Oligonucleotide | Sequence 5'-3' |
|---|---|
| 18s rRNA forward | 5'-GGCCCTTAAATAGCCCGGT-3' |
| 18s rRNA reverse | 5'-TGAGCCGATAGTCCCCCTAA-3' |
| Probe | 6-FAM-AGCCAGCGGCCCGCAAATG-MGB-3' |

### 2.2.22 Ligand Tracer Technology

A549 cells were seeded in one area of cell culture dish (Nunclon™, Thermoscientific, Germany) at concentration of $5\times10^5$ cells/ml and incubated for 16 h at 37 °C with humidified atmosphere of 5% (v/v) $CO_2$. The plates were positioned obliquely at a height of about 1.5 cm overnight so that the cells are grown on one part of plate. After 16 h, the cells were

infected with *A. fumigatus* spores and again incubated for a period of 12 h. The uptake measurements were performed using Ligand Tracer white (Bjorke, H., and Andersson, K., 2006 Bjorke, H., and Andersson, K., 2006) (Ridgeview Instruments AB, Uppala, Sweden).

The fungus grown on cells in one part of the dish acted as the sample area and the part of dish without cells acted as reference area for the measurement. Before assembling of the dish on the Ligand Tracer device, the plate was rinsed thoroughly twice with 5 ml of PBS. The dish was placed in the Ligand Tracer apparatus and the tracer solution, diluted in 4 ml of F-12K medium, was added. The measurement was run at 37 °C over approximately 70 min to determine uptake and over 30 min to determine retention of radio-tracer. Ligand tracer 1.0.2 software was used to analyze the uptake of tracer. The run was performed in uptake/retention mode with appropriate settings of decay correction, experimental time scale of minutes, 3+3 (opposite side) measure points, time of 4 s for detection of each stop and data collection frequency of one a minute. Plot settings were max (peak) / min (background). After the run, results were evaluated with “perimeter trace” which is revealed by the highest signal on point position 3 (mid point) with an equal distribution of the fungus. Resulting data were displayed in TraceDrawer 1.3 software.

### 2.2.23 Statistical analysis

The data represent at least three sets of independent experiments and are presented as mean ± standard deviation. Statistical analysis of data was conducted using unpaired Mann-Whitney U test and $p$ values ≤ 0.05 were considered significantly different and $p$ values of ≤ 0.01 were regarded as highly significant.

# 3 Results

## 3.1 ..Interaction of *A. fumigatus* conidia with alveolar epithelial cells

Upon inhalation of *A. fumigatus* conidia, the alveolar and bronchial surfaces are the primary sites of interaction of fungal and host cells. Besides being merely a physical barrier to the invading pathogenic organisms, these cell layers are also capable of phagocytosing the conidia and stimulate an immune response (Balloy *et al*; 2008. Wasylnka and Moore; 2002, 2003) though not as efficiently as primary phagocytes, like macrophages. In phagocytes like macrophages, conidia exert cytoprotective effect and sustain inside the cells (Volling et al., 2011). Since epithelial cells display reduced phagocytic activity and are the first barriers to invading conidia, it is reasonable to assume that some conidia might be able to find a reservoir within these cells and survive for some time within them. Such conidia could become the source of infection as soon as the host's immune system is impaired. Thus, the persistence of *A. fumigatus* conidia in lung epithelial cells was investigated and the factors involved in conidial survival were examined. The human alveolar epithelial cell line A549 was used as model system in all experiments, unless and otherwise stated.

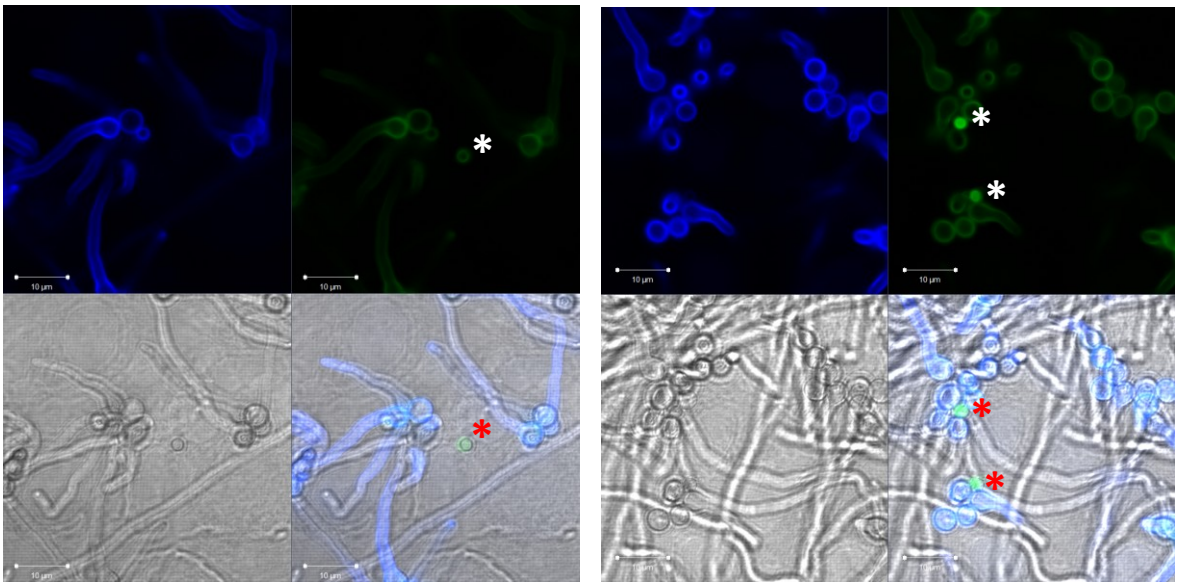
### 3.1.1 Uptake of *A. fumigatus* conidia by alveolar epithelial cells

#### 3.1.1.1 *A. fumigatus* conidia are phagocytosed by alveolar epithelial cells

Since epithelial cells are regarded as non-professional phagocytes, at first, how efficiently these cells are able to phagocytose *A. fumigatus* conidia was tested. The processing of conidia by epithelial cells was followed for a time period of up to 24 hours. Before infecting the cells with conidia, the viability of conidia was determined by colony forming units. About 97%-99% of conidia were viable at the time of infection of A549 epithelial cells. Internalization of conidia was quantified within cells by differential staining of intracellular and extracellular conidia using fluorescence microscopy (Mech et al., 2011). Based on this discrimination of the localisation of conidia, it was observed that *A. fumigatus* conidia were phagocytosed by A549 lung epithelial cells. After 12 h and 24 h, about 96 % of wild-type conidia had germinated (Fig 8A). Interestingly, about 4 % of spores had not germinated after 24 h and were still found inside the cells (Fig. 9 A, B). About 3 % of wild-type conidia were

present within epithelial cells after 6 h (Fig 9B). Hence, the conidia reside inside epithelial cells even for prolonged incubation times up to 24 h.

**(A)**



**(B)**

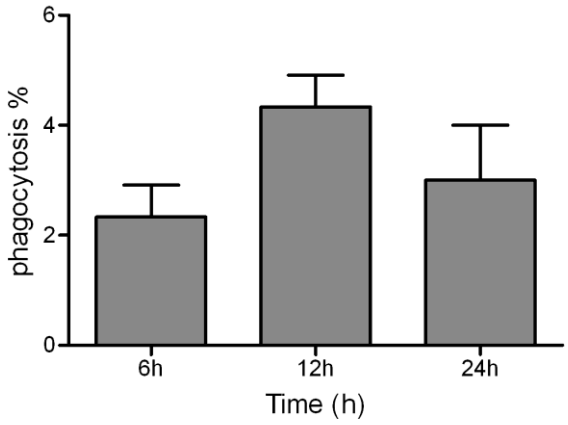



**Figure 9. Phagocytosis of *A. fumigatus* wild-type conidia by A549 lung epithelial cells.**
A549 cells were exposed to CEA10 conidia for 6, 12 and 24 hours. **A**) The microscopic images show the presence of non-germinated conidia inside epithelial cells (asterisk marks) after 12 and 24 h. In each image, phagocytosed conidia stained only green, whereas non-phagocytosed conidia stained blue and green. Upper left square: blue layer (calcolflour white), extracellular conidia and mycelia. Upper right square: green layer (FITC), all conidia. Lower left square: unstained cells; lower right square: overlay showing all conidia and cells. Bar scale: 10 µm. **B**) The phagocytosis rate was determined as the number of cells with at least one internal conidium per 100 cells per field (mean ± s.d., n= 3).

### 3.1.1.2 Uracil auxotrophic strain CEA17 is used to follow the fate of intracellular conidia.

To monitor the fate and processing of intracellular conidia in epithelial cells and to prevent the interference of germinating conidia, a uracil auxotrophic strain (CEA17) that is unable to germinate without uracil, was used (D'Enfert et al., 1996). *A. fumigatus* strain CEA17 contains a non functional *pyrG* gene encoding a gene of the uracil biosynthesis pathway. The co-incubation time of cells with these conidia could be extended from 6 h up to 24 h,

thereby; the survival of spores inside the cells could be monitored without interference of outgrowing mycelia which quickly covers the cell culture.

Before interaction of CEA17 uracil-auxotrophic strain with epithelial cells, the nature of these conidia was evaluated and compared with wild-type conidia. The wild-type strain CEA10 and the uracil-auxotrophic strain CEA17 showed same size of conidia when observed microscopically (Fig 10 A, B). Also, no obvious change in morphology of CEA17 strain was observed, when supplemented with uracil/uridine (Fig 10 C, D). In addition, CEA17 conidia showed the same production of melanin which was visualized by the presence of grayish green pigment (Fig 10 E). Thus, uracil auxotrophic strain CEA17 was used to follow the fate of *A. fumigatus* conidia inside epithelial cells.

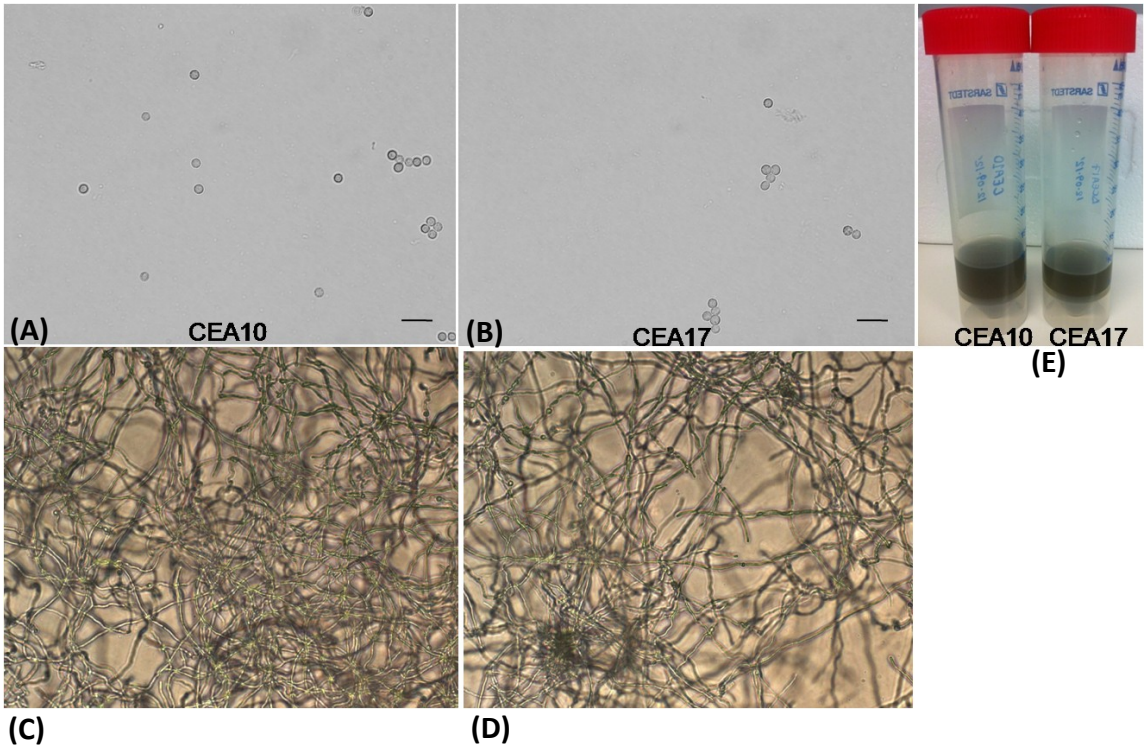



**Figure 10. Macroscopic and microscopic analyses of wild-type (CEA10) and uracil-auxotrophic (CEA17) conidia**

Microscopical analysis of **A)** wild-type strain CEA10 and, **B)** uracil-auxotrophic strain CEA17 show similar conidial size. The morphology of **C)** wild type strain CEA10 and **D)** uracil auxotrophic strain CEA17 when supplemented with uracil/uridine, show similar mycelial growth. **E)** Both CEA10 and CEA17 conidia produce melanin and are grayish green in color. Size of bar: 10 µm

### 3.1.1.3 *A. fumigatus* conidia reside inside epithelial cells

Uracil auxotrophic strain CEA17 was incubated with A549 cells and the phagocytosis rate was observed up to 24 h using confocal microscopy. The FITC labeled conidia were counterstained with calcoflour white to differentiate the intracellular conidia from extracellular conidia (Fig 11). Furthermore, to follow the internalization of wild-type conidia by epithelial cells without germinating into mycelia, the formaldehyde fixed conidia of wild-type *A. fumigatus* strain were used.

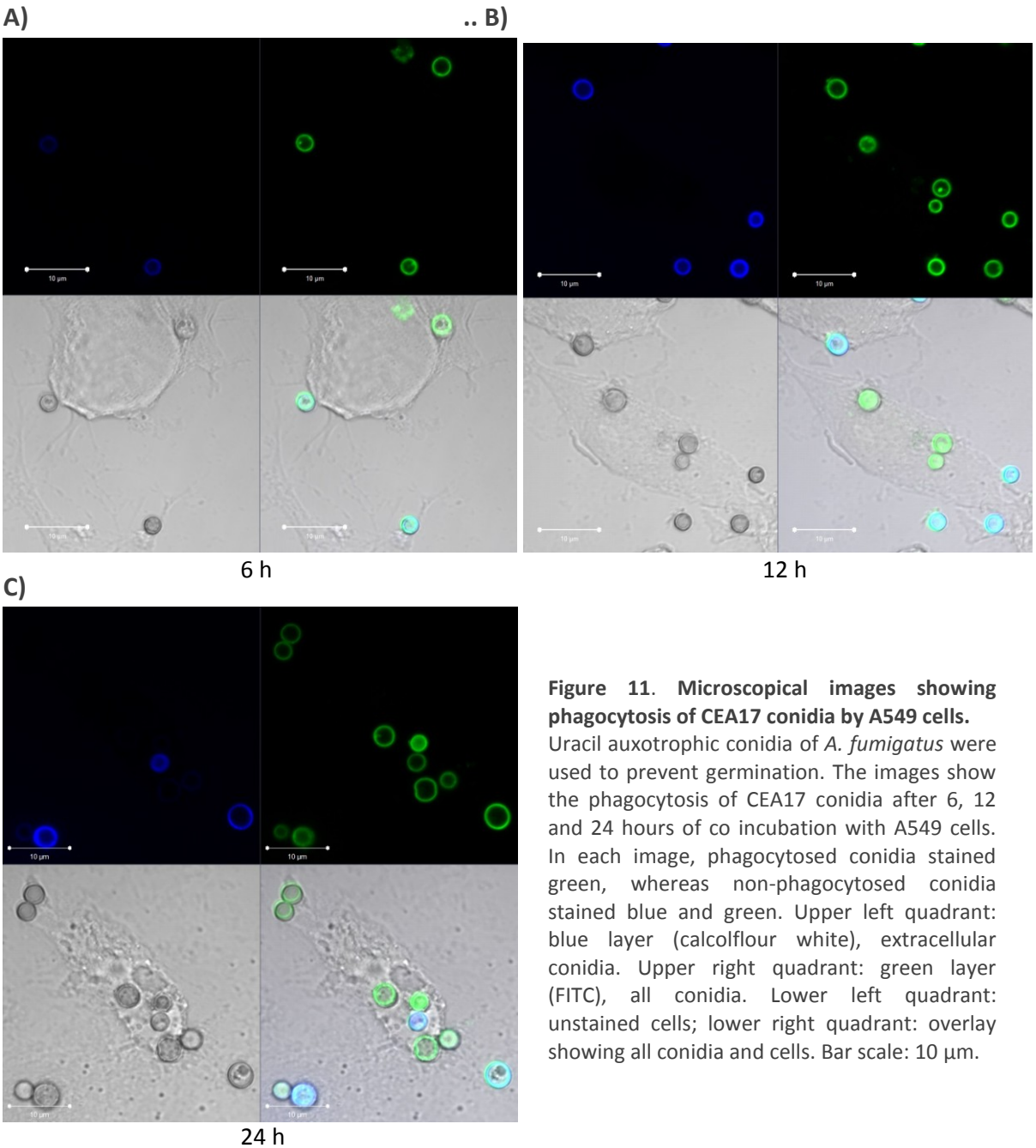



**Figure 11. Microscopical images showing phagocytosis of CEA17 conidia by A549 cells.** Uracil auxotrophic conidia of *A. fumigatus* were used to prevent germination. The images show the phagocytosis of CEA17 conidia after 6, 12 and 24 hours of co incubation with A549 cells. In each image, phagocytosed conidia stained green, whereas non-phagocytosed conidia stained blue and green. Upper left quadrant: blue layer (calcolflour white), extracellular conidia. Upper right quadrant: green layer (FITC), all conidia. Lower left quadrant: unstained cells; lower right quadrant: overlay showing all conidia and cells. Bar scale: 10 µm.

After 6 h of co-incubation with alveolar epithelial cells, CEA17 conidia showed the percentage of phagocytosis (Fig 12) similar to resting wild-type CEA10 conidia (< 10 %) (Fig 9). After 12 h, the phagocytosis rate of CEA17 conidia was 30 %, which increased to 47 % after 24 h (Fig. 12). The conidia fixed by *p*-formaldehyde were taken up more efficiently as compared with the uracil auxotrophic strain. Conidia were phagocytosed by epithelial cells and remained inside these cells. The results show that *A. fumigatus* conidia are internalized by epithelial cells and are able to reside inside these cells for longer time periods of up to 24 hours.

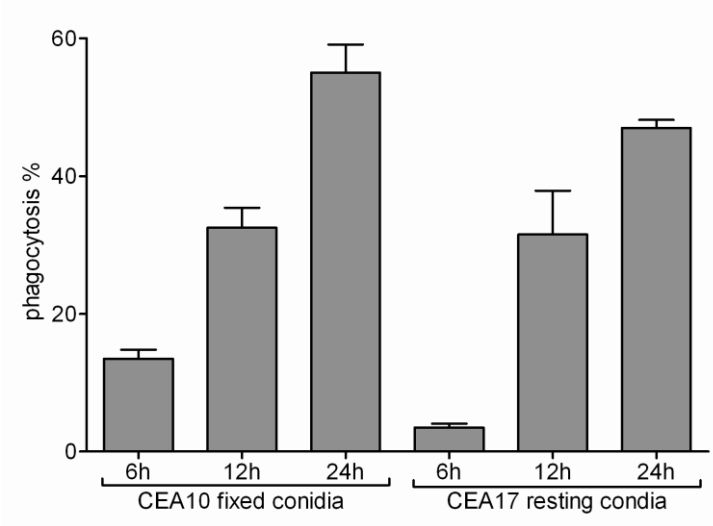



**Figure 12**. **Phagocytosis rate of formaldehyde fixed wild-type CEA10 and uracil auxotrophic CEA17 conidia by A549 lung epithelial cells.**
A549 cells were incubated with uracil auxotrophic conidia CEA17 and formaldehyde fixed CEA10 conidia. The phagocytosis rate was calculated after 6, 12 and 24 hours of co- incubation and determined as the number of cells with at least one internal conidium per 100 cells per field (mean ± s.d., n=4).

### 3.1.1.4 *A. fumigatus* conidia survive inside epithelial cells

By microscopical analyses of conidial interaction with epithelial cells, it was observed that *A. fumigatus* conidia are able to reside inside these cells. The next question was whether the conidia present inside these cells were viable or dead. To determine the viability of internalized conidia, a nystatin protection assay was applied (Wasylnka and Moore, 2002). The fungicide nystatin was added to cell cultures co-incubated with CEA17 conidia. The fungicide only killed extracellular conidia without affecting the viability of cells, and after lysing the cells, the number of conidia surviving within cells was calculated by counting the number of colony forming units.

The results showed that the internalized conidia are viable up to 24 h. After 6 h of co-culture of CEA17 conidia with epithelial cells, the percentage of internalized surviving conidia was about 10% which increased to about 15% and 25% after 12 and 24 h, respectively (Fig 13). This led to the conclusion that *A. fumigatus* conidia not only reside inside epithelial cells but are also able to survive within these cells for up to 24 h.

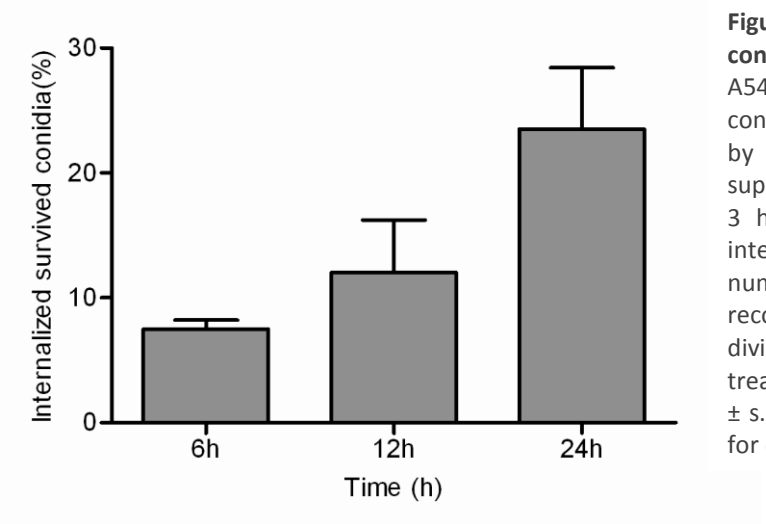



**Figure 13. Survival of *A. fumigatus* conidia within epithelial cells.**
A549 cells were incubated with CEA17 conidia for 6, 12 and 24 hours followed by treatment with nystatin-supplemented or nystatin-free media for 3 h. At each time point, the rate of internalization was determined as the number of colony forming units recovered from nystatin treated cells divided by the number from control-treated cells. Values represent the mean ± s.d. of three independent experiments for each time point.

### 3.1.1.5 Absence of DHN melanin reduces the recognition and uptake of *A. fumigatus* conidia by epithelial cells.

Resting conidia of *A. fumigatus*, which are constantly inhaled by humans, are covered by dihydroxynaphthalene (DHN) melanin. Since fixed pigmented conidia were phagocytosed efficiently by epithelial cells, it was investigated whether DHN melanin plays a role in uptake of conidia by epithelial cells. Therefore, the phagocytosis rate of melanised conidia and non-melanised conidia was compared. For this purpose, a uracil auxotrophic *pksP* mutant strain was generated, which lacks the central enzyme for synthesis of DHN melanin. As a result, conidia of this mutant strain, designated CEA17*pksP*, lack DHN melanin and are unable to germinate in absence of uracil (Fig 14 A). Additionally, the uptake of resting formaldehyde fixed melanised spores of wild type strain by A549 cells was further compared with non-melanised *pksP* spores (Jahn et al., 1997; Langfelder et al., 1998) derived from wild type strain.

The formaldehyde fixed *pksP* conidia showed about 4-fold decrease in phagocytosis rate after 24 h of co-incubation when compared with wild-type CEA10 conidia (Fig. 14 B). Resting live conidia of strain CEA17*pksP* showed a phagocytosis rate of 10 % and 19 % after 12 and

24 h of co-incubation with A549 cells, respectively, whereas the phagocytosis rate of resting live CEA17 conidia at these time points was about 37% and 48% (Fig. 14 C). Thus, after 12 and 24 h, a reduced phagocytosis rate was determined for non-pigmented white conidia compared to melanised conidia.

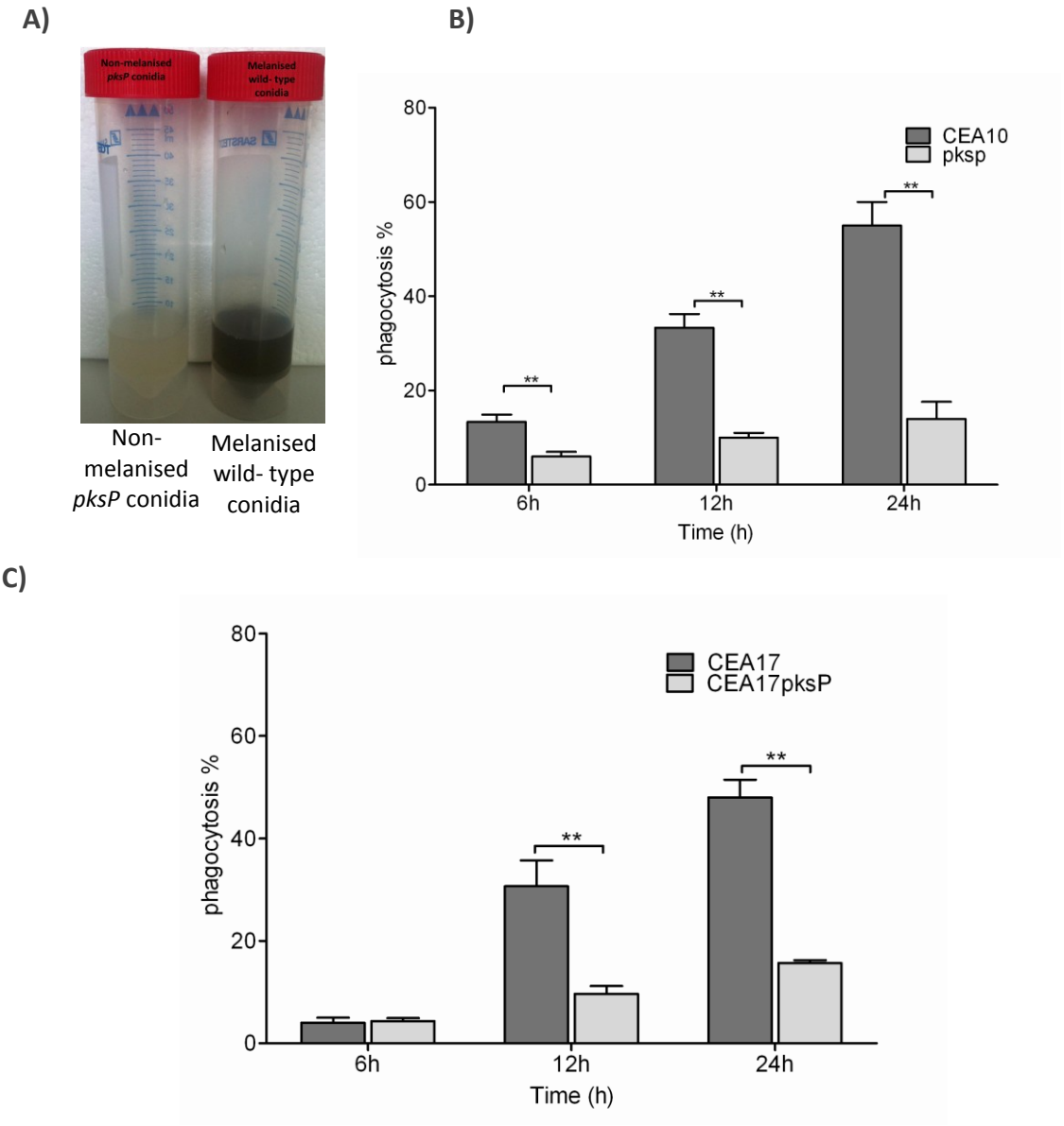



**Figure 14**. **Macroscopical analysis and phagocytosis rate of melanised and non-melanised *A. fumigatus* conidia by A549 lung epithelial cells**

**A)** Melanised CEA17 conidia and and non-melanised CEA17pksp conidia in *Aspergillus* minimal media supplemented with uracil. Phagocytosis was monitored by differential staining of conidia and the phagocytosis rate was determined as the number of cells with at least one internalized conidium per 100 cells per field. A549 cells were exposed to **A**) resting fixed conidia of CEA10 and strain *pksP* and, **B**) resting live conidia of CEA17 and CEA17pksP for 6, 12 and 24 hours. Values represent the mean ± s.d. of three independent experiments. (*, $P \leq 0.05$; **, $P \leq 0.01$, calculated by Mann-Whitney U test).

### 3.1.1.6 The uptake of *A. fumigatus* conidia by epithelial cells differs from alveolar macrophages

Previously it was shown that macrophages show entirely different phagocytosis behaviour, *i.e.*, *pksP* mutant conidia are far more efficiently phagocytosed than melanised wild-type conidia (Jahn et al., 1997). Therefore, MHS macrophages were tested under the same conditions and their phagocytic behaviour was compared to epithelial cells.

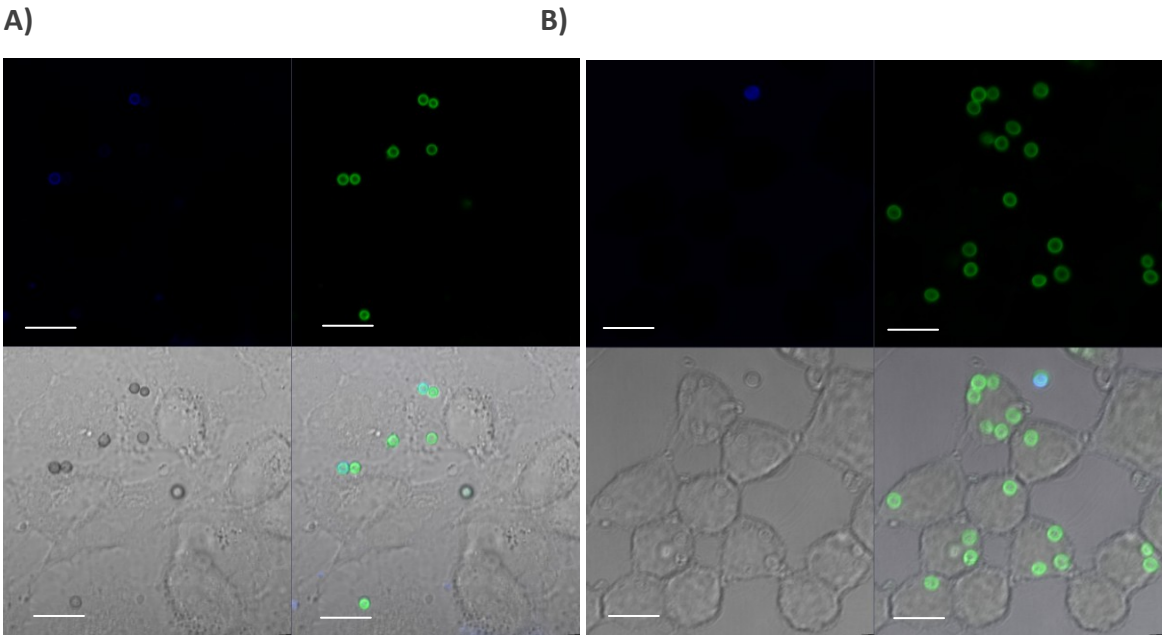



**Figure 15. Microscopical images of phagocytosis of *A. fumigatus* conidia by A549 lung epithelial cells and MHS macrophages.**
**A)** A549 epithelial cells with intra- and extra-cellular *A. fumigatus* conidia. **B)** MHS macrophages with intra- and extra-cellular *A. fumigatus* conidia. Phagocytosis was observed by differential staining of conidia. In each image: Upper left square: extracellular conidia (blue layer, calcoflour white); upper right square: green layer (FITC), all conidia. Lower left square: unstained cells; lower right square: overlay showing all conidia and cells. Bar scale: 10 µm.

As shown previously and unlike epithelial cells, macrophages showed increased uptake of *pksP* mutant conidia compared to wild-type conidia. This was not the case when epithelial cells were investigated (Fig 15, Fig 16). This shows that the uptake of conidia by epithelial cells differs from other immune cells. Taken together, melanised conidia are recognized and taken up better compared to non-melanised conidia by epithelial cells and are able to reside within the cellular compartments of epithelial cells.

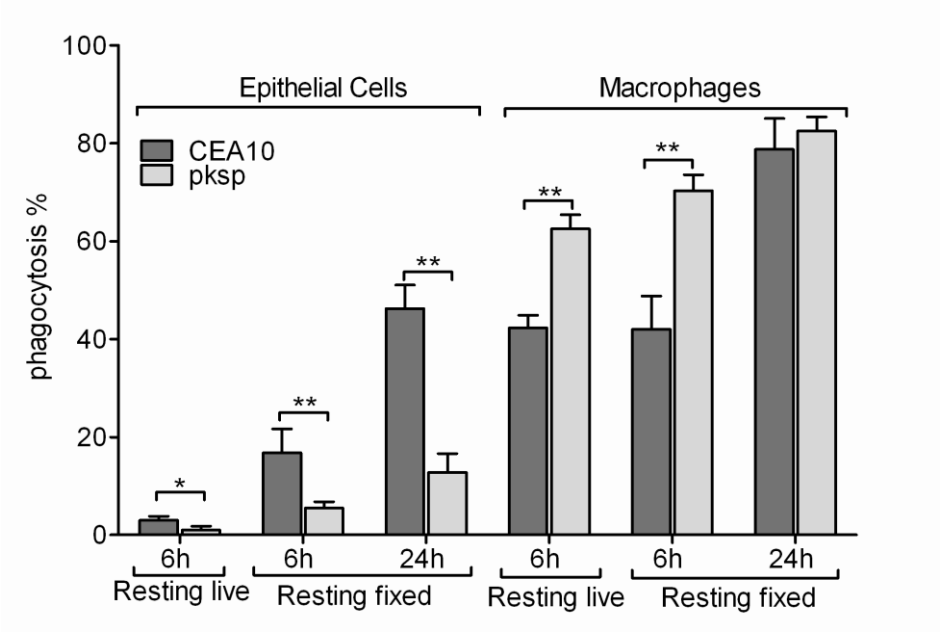



**Figure 16. Comparison of phagocytosis of melanised and non-melanised *A. fumigatus* conidia by A549 lung epithelial cells and macrophages.**
The phagocytosis rate of melanised and non-melanised conidia by epithelial cells was compared with macrophages. A549 cells and MHS cells were exposed to resting fixed conidia of CEA10 and *pksP* and resting live conidia of CEA17 and CEA17pksP, for 6 and 24 hours. The phagocytosis rate was determined as the number of cells with at least one internalized conidium per 100 cells per field. Values represent the mean ± s.d. of three independent experiments. (*, $P \leq 0.05$; **, $P \leq 0.01$, calculated by Mann-Whitney U test).

### 3.1.1.7 Co-culture of melanised and non-melanised conidia also show increased uptake of melanised conidia by epithelial cells

After comparing the phagocytosis rate of melanised and non-melanised conidia by epithelial cells and macrophages, the competitive behaviour of melanised and non melanised conidia, when inoculated together with epithelial cells, was checked. The uptake of conidia by epithelial cells was determined by differential staining of conidia using confocal microscopy. The melanised wild type conidia were stained with FITC while *pksP* conidia were not stained and incubated simultaneously with epithelial cells. After 6 h, the extracellular conidia were counterstained with calcoflour white. The intracellular melanised conidia stained only green while as the extracellular melanised conidia stained green and blue. Also, the intracellular *pksP* conidia remained unstained while as the extracellular *pksP* conidia stained only blue (Fig 17 A, B, C). Besides resting live wild-type conidia (Fig 17 A, B), the formaldehyde fixed melanised and non-melanised conidia were also used (Fig 17 C).

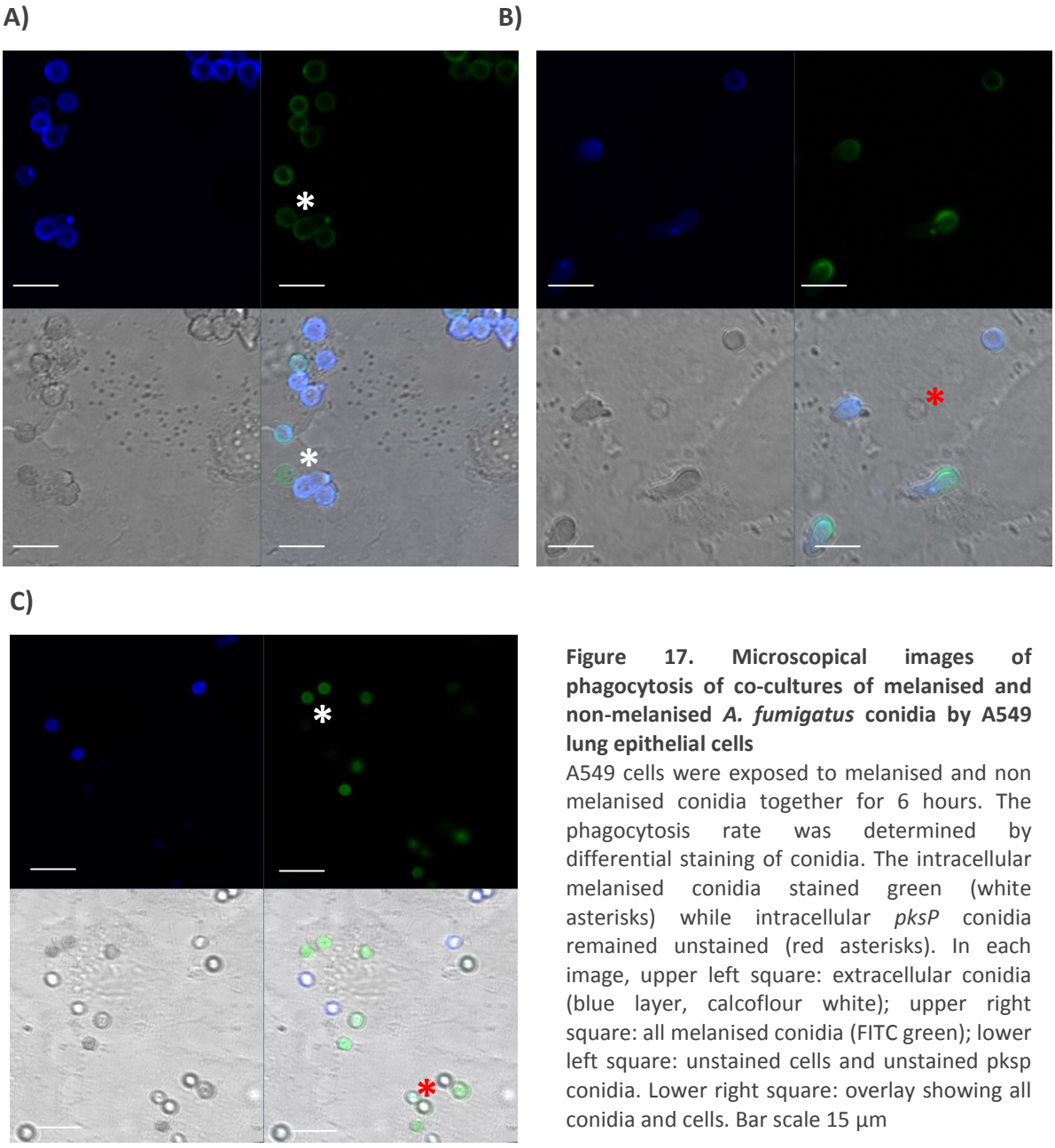



**Figure 17. Microscopical images of phagocytosis of co-cultures of melanised and non-melanised *A. fumigatus* conidia by A549 lung epithelial cells**

A549 cells were exposed to melanised and non melanised conidia together for 6 hours. The phagocytosis rate was determined by differential staining of conidia. The intracellular melanised conidia stained green (white asterisks) while intracellular *pksP* conidia remained unstained (red asterisks). In each image, upper left square: extracellular conidia (blue layer, calcoflour white); upper right square: all melanised conidia (FITC green); lower left square: unstained cells and unstained pksp conidia. Lower right square: overlay showing all conidia and cells. Bar scale 15 µm

Again, melanised conidia were taken up more efficiently than non melanised conidia. The resting conidia of the *pksP* mutant showed no uptake of conidia compared with melanised wild type conidia after 6 h of coinicubation with epithelial cells. The resting fixed conidia of wild type showed a more than 4 fold increase in uptake by epithelial cells compared to resting fixed conidia of the *pksP* mutant (Fig 18).

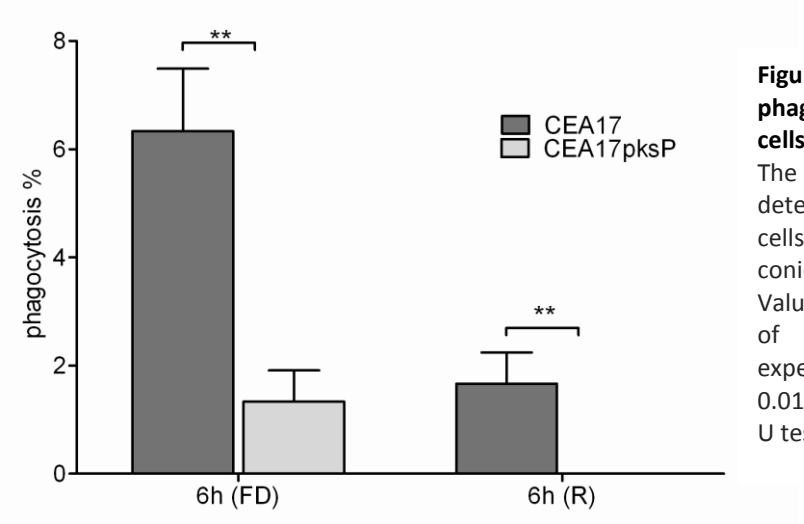



**Figure 18. Impact of melanin on phagocytosis of conidia by A549 cells**
The phagocytosis rate was determined as the number of cells with at least one internalized conidium per 100 cells per field. Values represent the mean ± s.d. of three independent experiments. (*, $P \leq 0.05$; **, $P \leq 0.01$, calculated by Mann-Whitney U test).

### 3.1.1.8 Melanised *A. fumigatus* conidia survive within alveolar epithelial cells

The results obtained so far showed that melanised conidia are able to survive inside epithelial cells. Since *pksP* conidia showed decreased phagocytosis rate, the viability of these non-melanised conidia inside epithelial cells was checked. Again, the viability of internalized conidia was analyzed by nystatin survival assay as described in section 3.1.1.4. As shown in Fig. 19, after 6 h there was no significant difference in internalized survival rates between phagocytosed conidia of CEA17 and CEA17*pksP*. However, after 12 h and 24 h, the survival rate of phagocytosed CEA17 conidia increased up to 25 %, whereas the survival rate of non-melanised CEA17*pksP* conidia leveled at 10 % at all time points. Thus, the nystatin survival assay indicated that melanised conidia of *A. fumigatus* are able to reside and survive within epithelial cells and that DHN melanin plays a major role for survival.

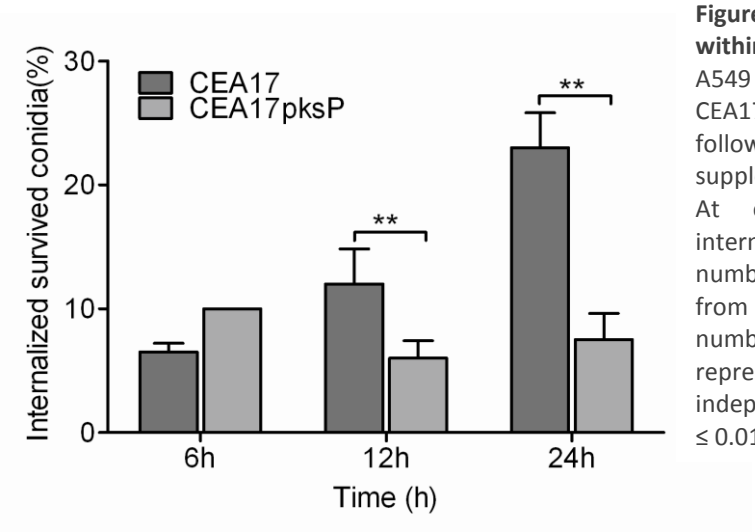



**Figure 19. Survival of *A. fumigatus* conidia within epithelial cells.**
A549 cells were incubated with CEA17 and CEA17pksP conidia for 6, 12 and 24 hours followed by treatment with nystatin-supplemented or nystatin-free media for 3 h. At each time point, the rate of internalization was determined as the number of colony forming units recovered from nystatin treated cells divided by the number from control-treated cells.. Values represent the mean ± s.d. of three independent experiments. (*, $P \leq 0.05$; **, $P \leq 0.01$, calculated by Mann-Whitney U test).

### 3.1.2 Inhibition of apoptosis of epithelial cells by *A. fumigatus* conidia.

#### 3.1.2.1 *A. fumigatus* conidia inhibit the extrinsic apoptosis pathway in epithelial cells

Inhibition of apoptosis provides a survival advantage to the pathogen within the host (Faherty and Maurelli, 2008). Since *A. fumigatus* conidia were able to reside and survive inside epithelial cells without affecting the viability of cells, thus the effect of *A. fumigatus* conidia on the apoptotic process of A549 epithelial cells was investigated. Apoptotic cells are characterized by morphological changes like cell shrinkage, membrane blebbing, DNA fragmentation and formation of apoptotic bodies. To analyze the effect of *A. fumigatus* conidia on apoptosis of epithelial cells, the extent of DNA fragmentation in infected cells was first monitored by measuring the hypodiploid DNA content of cells by propidium iodide staining using flow cytometry. The hypodiploid subG1 DNA content represents the percentage of apoptotic cells in the sample (Fig 20). The extrinsic, receptor mediated, apoptosis pathway was initiated by TNF-α (Bhardwaj and Aggarwal, 2003; Krammer et al., 2007; Rath and Aggarwal, 1999), together with the mRNA translation inhibitor cycloheximide (CHX), which suppresses survival signals induced by engagement of TNF-receptors (Higuchi et al., 1995). The treatment of TNF-α alone did not trigger apoptosis in A549 cells.

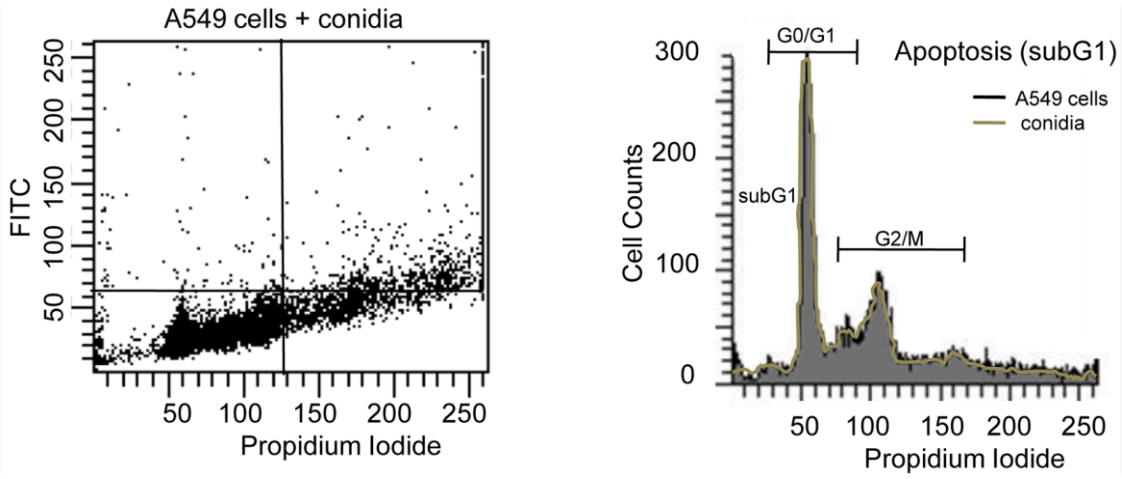



**Figure 20**. **Hypodiploid subG1 DNA content by propidium iodide staining using flow cytometry**
A549 cells were exposed to *A. fumigatus* conidia before induction of apoptosis with TNF-α and cycloheximide (CHX) for 6, 8 and 10 hours. **(A)** The upper right quadrant of the cytogram represents A549 cells with phagocytosed conidia (PI+/FITC+), while FITC-labbeled conidia are shown in the upper left quadrant (FITC+/PI-). The lower right quadrant represents only A549 cells (PI+/FITC-). The fluorescence of 10,000 cells was assessed in each case. **(B)** DNA profiles of A549 cells with *A. fumigatus* conidia and treated with TNF-α/CHX. Apoptosis rate was measured by propidium iodide FACS-analysis (subG1 fraction, apoptotic cells with a DNA content < 2n).

At the time points 8 h and 10 h, A549 cells were challenged with FITC-labeled CEA17 conidia at an MOI of 0.5 for 2h and 4 h, respectively, before TNF-α/CHX was applied and then incubated for another 6h. For 6h time point, the apoptotic inducer was added simultaneously with *A. fumigatus* conidia to the cells. A549 cells with *A. fumigatus* conidia and without apoptotic stimulus were also analyzed for hypodiploid (subG1) DNA content.

After exposure of A549 cells to TNF-α/CHX for 6 h, apoptosis was triggered by the extrinsic pathway in 23 % of cells as measured by hypodiploid DNA (subG1) content, while in the untreated control sample (A549 cells only), apoptosis was not induced. Apoptosis was also not induced when the cells were solely co-incubated with *A. fumigatus* conidia without TNF-α/CHX. Remarkably, TNF-α/CHX-induced apoptosis was nearly completely inhibited in cells that were pre-infected with *A. fumigatus* conidia. The anti-apoptotic effect of *A. fumigatus* conidia was even detectable after 12 h and 24 h of co-incubation (data not shown). At late time spans, the percentage of apoptotic cells decreased significantly compared to the cells treated solely with TNF-α/CHX (Fig. 21). However, an increase in the pre-incubation time of A549 cells with *A. fumigatus* conidia prior to TNF-α/CHX treatment decreased the amount of apoptotic cells. This shows that *A. fumigatus* conidia are able to inhibit TNF-α/CHX induced apoptosis in A549 lung epithelial cells.

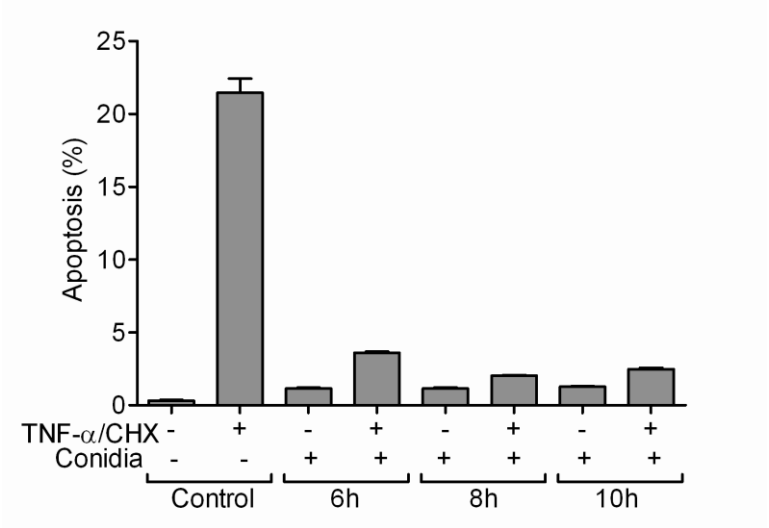



**Figure 21**. **Inhibition of apoptosis of epithelial cells by *A. fumigatus* conidia**
The percentage of apoptotic cells was determined by analyzing the hypodiploid DNA (subG1) content of cells which was measured by propidium iodide staining and flow cytometry(mean ± s.d., n=5).

### 3.1.2.2 *A. fumigatus* conidia inhibit the extrinsic apoptosis pathway of epithelial cells by interfering with caspase-3 processing.

Caspases play an important role in the process of apoptosis. The activation of caspases is responsible for the morphological changes, like nuclear fragmentation, associated with apoptosis. Caspase-3 is a common link between intrinsic and extrinsic apoptotic pathways. To further investigate the inhibition of apoptosis by *A. fumigatus* conidia in epithelial cells, the effect of *A. fumigatus* conidia on the processing of effector caspase-3 was analyzed by western blots.

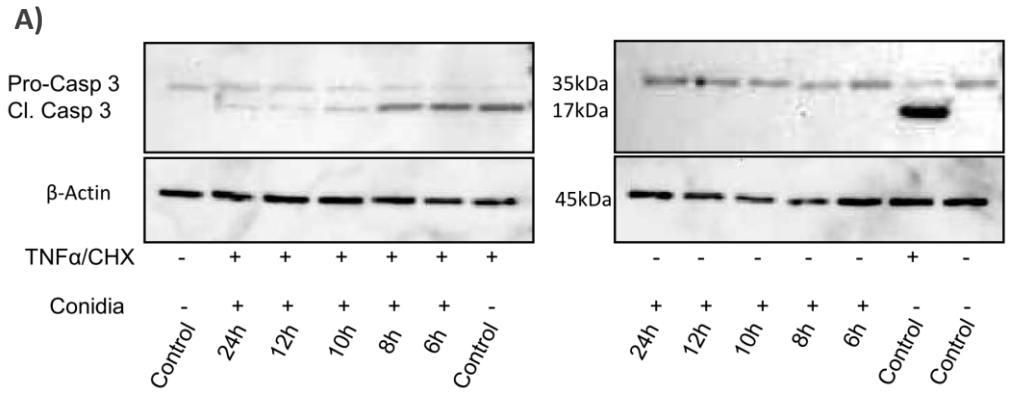



**Figure 22. Analysis of caspase-3 processing in TNF-α/CHX treated A549 cells by Western blots.**
A549 cells were pre-incubated with *A. fumigatus* conidia for 6, 8, 10, 12 and 24 hours before treatment with TNF-α/CHX. Control A549 cells, cells exposed to conidia in the presence or absence of apoptotic inducers and cells treated solely with apoptotic inducers were analyzed for pro-caspase-3 processing. Pro-caspase-3 and caspase-3 were detected by anti-caspase-3 antibody.

In TNF-α/CHX induced apoptosis in A549 cells, processing of inactive pro-caspase-3 (35 kDa) to active caspase-3 was detected after 6 h, indicated by the appearance of a 17 kDa band of active caspase-3 and the disappearance of the pro-caspase-3 band, whereas untreated A549 cells and cells exposed to *A. fumigatus* conidia without apoptotic inducer, contained the inactive pro-form of caspase-3 (Fig 22). A549 cells which were pre-infected with *A. fumigatus* conidia followed by treatment with TNF-α/CHX showed a drastic decrease in active caspase-3 (Fig 23). Moreover, with increase in the exposure of *A. fumigatus* conida to A549 epithelial cells, prior to TNF-α/CHX treatment, the levels of active caspase-3 decreased more. For example, A549 cells pre-incubated with *A. fumigatus* conidia for 18 h, followed by exposure to TNF-α/CHX for 6 h, showed lower intensity of active caspase-3 when compared with cells

exposed to conidia for 6 hours. Hence, *A. fumigatus* conidia inhibit TNF-α/CHX induced apoptosis of epithelial cells by inhibiting the processing of inactive pro-caspase 3 to active caspase 3.

**B)**

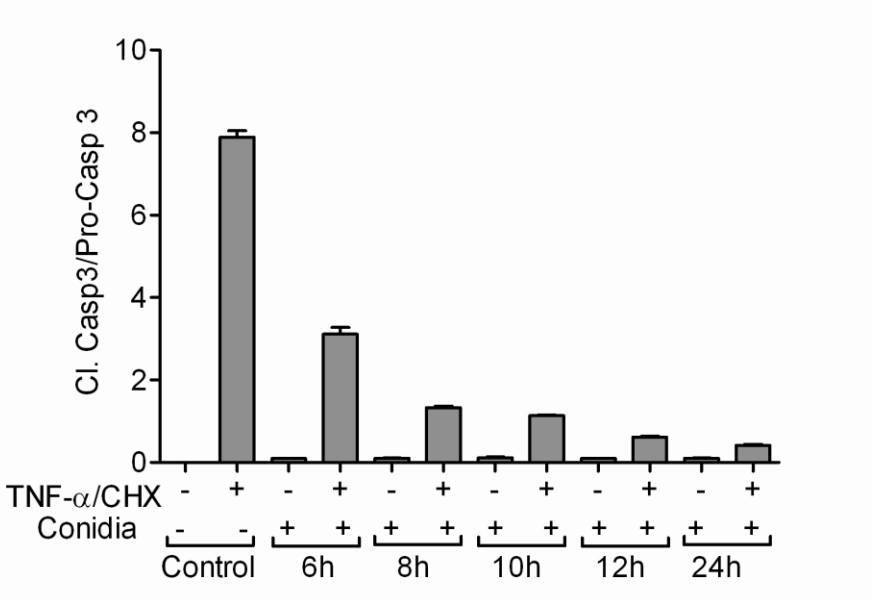



**Figure 23**. **Analysis of caspase-3 processing in TNF-α/CHX treated A549 cells and modulation of apoptosis by conidia**
The relative caspase-3 activity in each sample was calculated by dividing the values of the band intensities of active caspase-3 by that of pro-caspase 3 measured by western blot analysis (mean ± s.d., n = 3)

### 3.1.2.3 *A. fumigatus* conidia also inhibit intrinsic apoptosis of epithelial cells

Next, it was analysed whether *A. fumigatus* conidia are also able to inhibit the intrinsic apoptotic pathway. For that, apoptosis was induced in A549 epithelial cells by staurosporin (STS), a protein kinase inhibitor and a potent inducer of mitochondrial-mediated apoptosis (Banga et al., 2007; Harkin et al., 1998). To monitor the effect of *A. fumigatus* conidia on the STS-induced apoptotic pathway, the extent of DNA fragmentation was again analyzed by calculating the hypodiploid (subG1) DNA content. Furthermore, the processing of inactive pro-caspase-3 to active caspase-3 was analyzed by western blots.

When analyzed by propidium iodide staining for hypodiploid (subG1) DNA content of the cells, STS induced apoptosis in A549 cells to a very less extent (< 15%). However, pre-infection of A549 cells with *A. fumigatus* conidia exhibited a significant decrease in DNA fragmentation level (hypodiploid DNA content) when treated with STS. STS-untreated A549

cells showed no fragmentation of host DNA. A549 cells with *A. fumigatus* conidia and without STS treatment also showed no hypodiploid DNA fraction (Fig 24 A). However, when apoptosis was monitored by processing of caspase-3, no cleavage of procaspase-3 occurred on addition of STS to A549 cells (Fig 24 B). This adds to the above mentioned low DNA fragmentation during flow cytometry analysis, as effector caspases are important for DNA fragmentation.

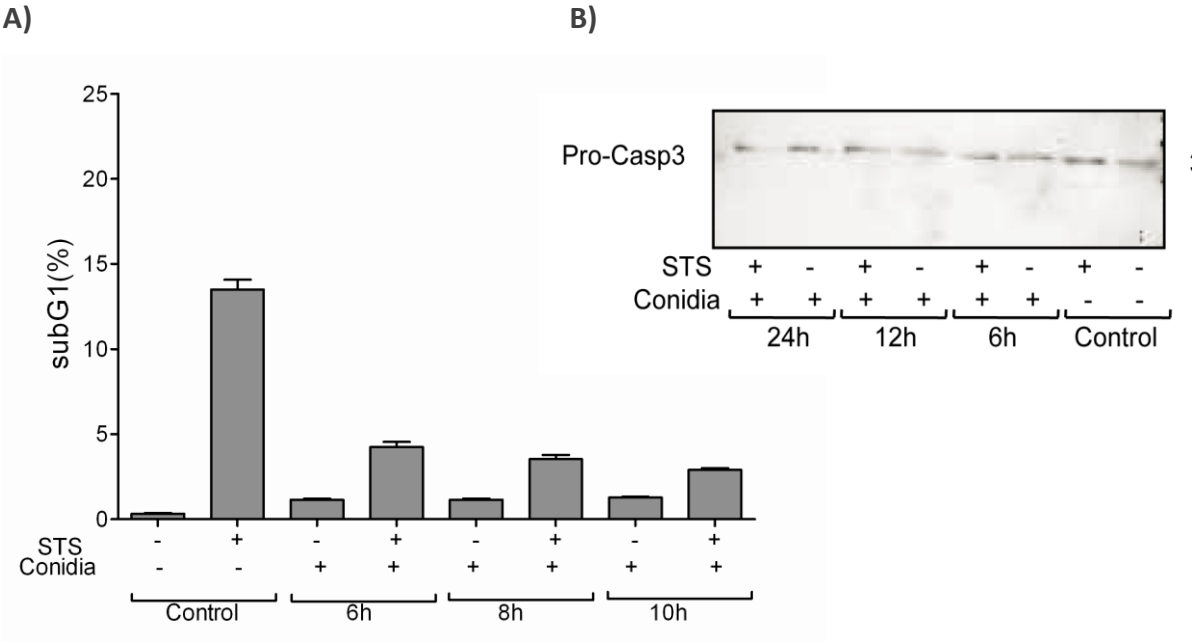
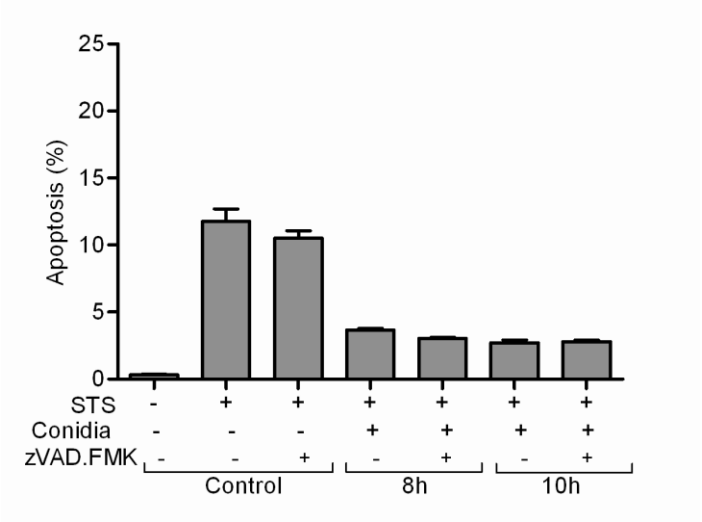



**Figure 24**. **Analysis of caspase-3 processing in STS treated A549 cells and modulation of apoptosis by conidia**
**A)** The relative caspase-3 activity in each sample was calculated by dividing the values of the band intensities of active caspase-3 by that of pro-caspase 3 measured by western blot analysis (mean ± s.d., n = 3). **B)** Western blots. A549 cells were pre-incubated with *A. fumigatus* conidia for 6, 8, 10, 12 and 24 hours before treatment with STS. Control A549 cells, cells exposed to conidia in the presence or absence of apoptotic inducers and cells treated solely with apoptotic inducers were analyzed for pro-caspase-

To further confirm that caspase-3 processing is not involved in STS induced apoptotic pathway in epithelial cells, the caspase inhibitor Z-VAD-FMK was added to the cells 1 hour before treating the cells with apoptotic inducers. The addition of the caspase inhibitor to STS induced cells did not alter apoptosis when analyzed for subG1 DNA content. Moreover, inhibition of STS-induced apoptosis by conidia did not increase when Z-VAD-FMK was added. This shows that induction of apoptosis by STS in epithelial cells does not involve caspase-3 processing (Fig 24 A). Furthermore, Z-VAD-FMK treated cells were also exposed to TNF/CHX. *A. fumigatus* conidia, like the caspase inhibitor Z-VAD-FMK, were able to inhibit apoptosis of

epithelial cells. Furthermore, Z-VAD-FMK had an additive effect on the inhibition of TNFα /CHX-induced apoptosis when pre-infected with *A. fumigatus* conidia (Fig 24 B). Due to non-involvement of effector caspase-3 in STS-induced apoptosis in A549 epithelial cells, all further studies were carried on with TNF-α/CHX induced apoptosis.

**A)**




**B)**

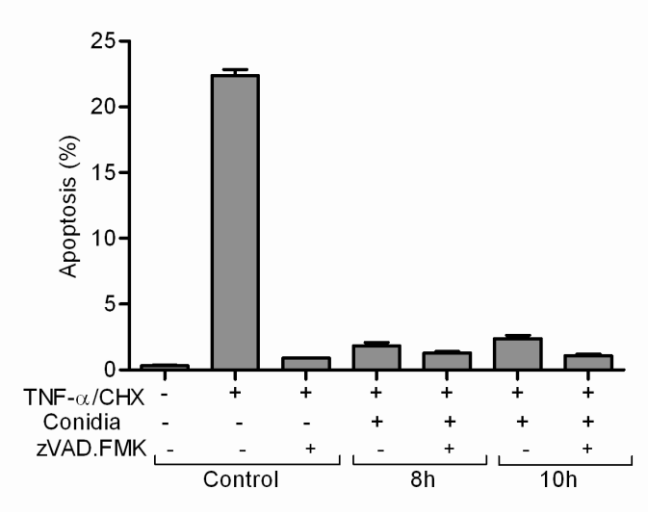



**Figure 24**. **Modulation of apoptosis by conidia and the caspase inhibitor Z-VAD-FMK.**
Z-VAD-FMK was added to the samples after exposure of A549 cells with *A. fumigatus* conidia one hour before the treatment with apoptotic inducers **A)** STS and **B)** TNF-α/CHX and analyzed for subG1 level of cells. The percentage of apoptotic cells was determined by analyzing the hypodiploid DNA (subG1) content of cells which was measured by propidium iodide staining and flow cytometry. DNA content analysis for subG1 level by flow cytometry gives the fraction of apoptotic cells in the samples at 8 and 10 hours (mean ± s.d., n = 3)

### 3.1.2.4 DHN melanin is essential for the anti-apoptotic effect of *A. fumigatus* conidia on epithelial cells

Based on the results that DHN melanin plays an important role in uptake of *A. fumigatus* conidia by epithelial cells, the effect of DHN melanin on the inhibition of apoptosis was analyzed. DHN melanin from *A. fumigatus* conidia was shown to play an important role in the sustained survival of infected macrophages (Volling et al., 2011) and to interfere with the

intracellular processing of conidia (Jahn et al 2002; Slesiona et al., 2012; Thywissen et al., 2011). Furthermore, DHN melanin of *A. fumigatus* has been shown to suppress host responses by reducing phagocytosis (Tsai et al., 1998) which was not the case for phagocytosis of *A. fumigatus* conidia by A549 epithelial cells (this study). To analyse whether DHN melanin is also involved in the inhibition of apoptotic processes in A549 cells, the impact of melanised conidia on inhibition of apoptosis was compared with that of non-melanised conidia. As shown in Figure 25, in contrast to pigmented conidia of CEA17, white CEA17*pksP* conidia did not inhibit TNF-α/CHX induced apoptosis in A549 cells. Analysis of DNA fragmentation levels of A549 cells with or without addition of TNF-α/CHX revealed lower hypodiploid (subG1) DNA content when co-incubated with melanised conidia compared to non-melanised conidia (Fig. 25).

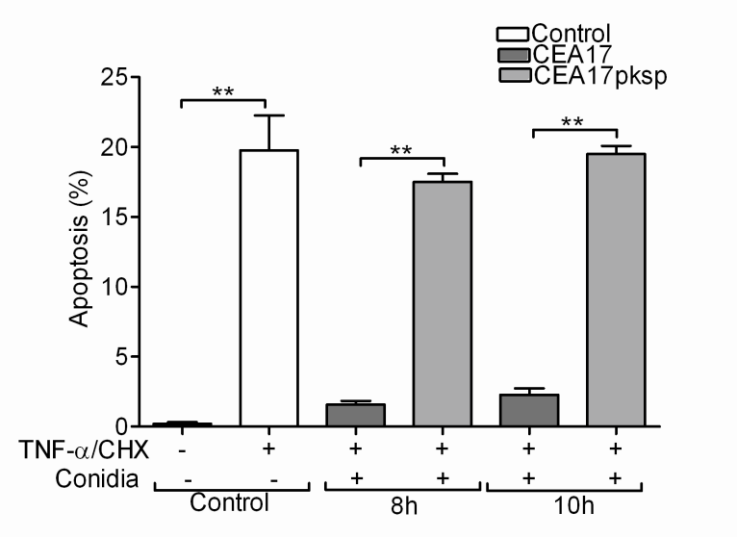
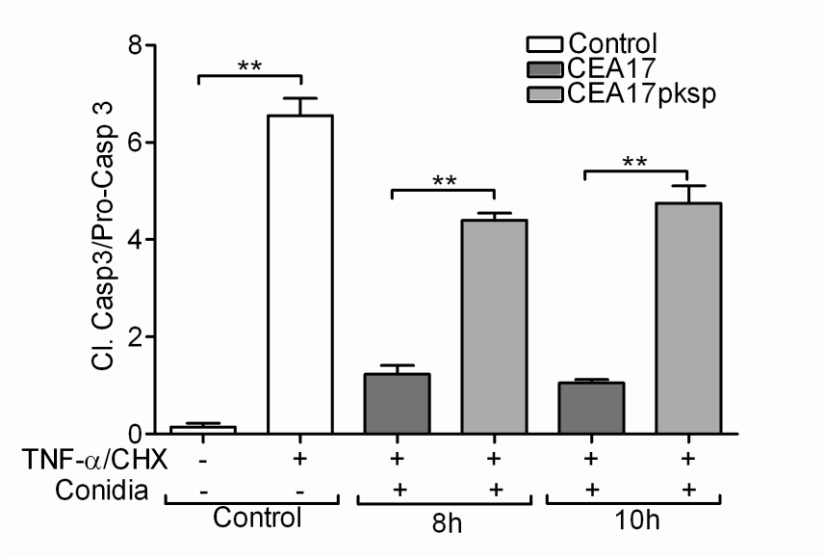



**Figure 25**. **Influence of melanin on apoptosis of A549 alveolar epithelial cells by flow cytometry**
A549 cells were challenged with wild-type CEA17 or CEA17*pksP* conidia before treatment with apoptotic inducers. DNA content was analyzed by flow cytometry for subG1 levels. Data were used to calculate the fraction of apoptotic cells. Values represent the mean ± s.d. of three experiments. (*, $P \leq 0.05$; **, $P \leq 0.01$, calculated by Mann-Whitney U test).

The impact of DHN melanin on the inhibition of apoptosis was further confirmed by western blot analysis of pro-caspase-3 processing during TNF-α/CHX induced apoptosis. The active caspase-3 levels were strongly reduced in cells co-incubated with melanized CEA17 conidia but not with non-pigmented CEA17*pksP* conidia prior to treatment with TNF-α/CHX (Fig 26 A). A549 cells with conidia and without addition of TNF-α/CHX did not show the presence of cleaved caspase-3 (Fig 26 B). These results are in agreement with flow cytometry analyses of

apoptotic cells showing that melanised conidia inhibit apoptosis of alveolar epithelial cells. Taken together, DHN melanin of *A. fumigatus* conidia plays an important role in inhibition of apoptosis of A549 epithelial cells.

**A)**




**B)**

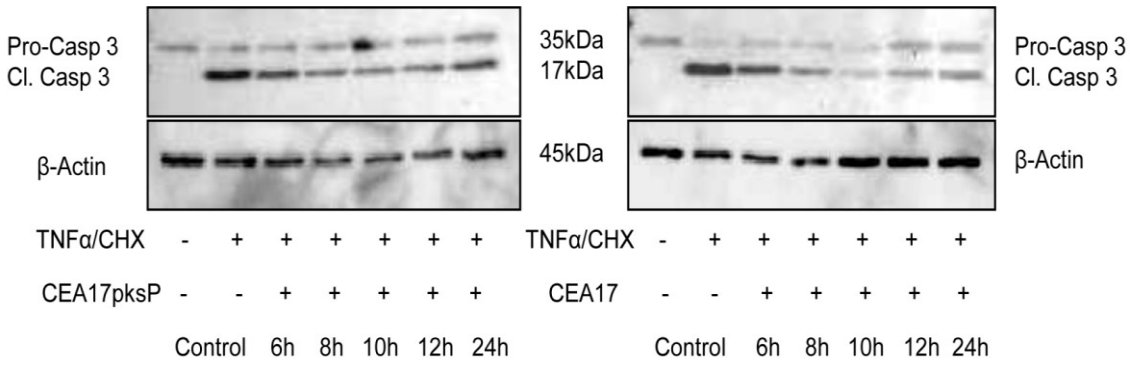



**Figure 26**. **Influence of melanin on apoptosis of A549 alveolar epithelial cells.**
**A**) A549 cells were challenged with CEA17 or CEA17*pksP* conidia before treatment with apoptotic inducers. DNA content was analyzed by flow cytometry for hypodiploid subG1 DNA levels. **B**) Western blot analysis of pro-caspase-3 processing after induction of apoptosis by TNF-α/CHX in presence or absence of CEA17 conidia or CEA17*pksP* conidia. Values represent the mean ± s.d. of three independent experiments. (*, $P \leq 0.05$; **, $P \leq 0.01$, calculated by Mann-Whitney U test).

### 3.1.2.5 Not only DHN melanin but other types of melanin also inhibit apoptosis in alveolar epithelial cells.

To investigate whether the anti-apoptotic effect is specific for DHN melanin or can also be mediated by other types of melanin, the apoptotic inhibitory effect of melanin isolated from *Sepia officinalis* (*S. o.* DOPA-melanin) and synthetic DOPA melanin was analyzed. Furthermore, DHN-melanin ghosts derived from wild-type conidia were tested to exclude that components other than DHN melanin were responsible for the observed effects. As a read out, TNF-α/CHX-induced apoptosis was determined by measuring pro-caspase-3 processing in A549 cells. As shown in Fig. 27, co-incubation of epithelial cells with all types of melanin strongly decreased the levels of processed pro-capase-3 upon induction by TNF-α/CHX. These data confirmed the inhibitory effect of melanin on apoptosis of epithelial cells and indicated that this effect can be caused by different types of melanin.

**A)** **B)**

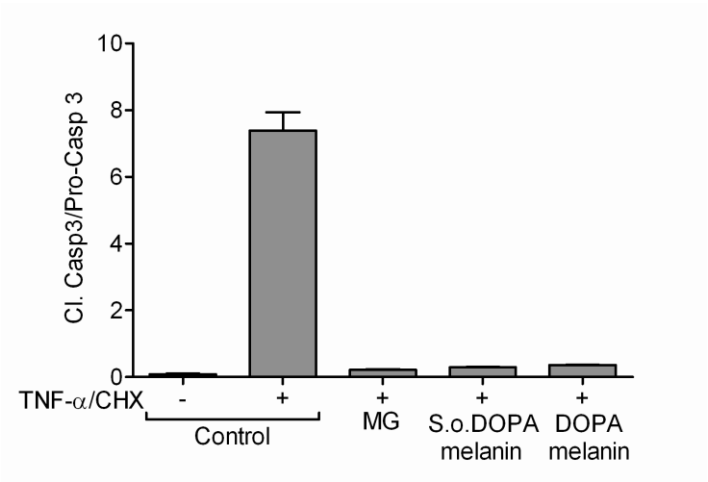



**Figure 27. Effect of different types of melanin on apoptosis.**
**A)** Electron micrograph of melanin ghost by Youngchim et al., 2003. **B)** Apoptosis was induced by TNF-α/CHX after A549 cells were exposed to DOPA melanin from *Sepia officinalis* (*S.o.* DOPA), synthetic DOPA melanin (DOPA) or DHN melanin ghosts (MG) prepared from *A. fumigatus*. The resulting effects on TNF-α/CHX induced pro-caspase-3 processing were analyzed by western blotting. Values represent the mean +/- s.d. of three independent experiments.

### 3.2. Dissemination of *A. fumigatus* after pulmonary infection in mice

The regular pathway for entry of *A. fumigatus* is via the inhalation of airborne conidia. In healthy, immune-competent individuals, the germinating conidia and hyphae are eliminated by host immune cells without occurrence of disease (Dagenais and Keller, 2009; Schaffner et al., 1982). In immunosuppressed patients, however, the mycelia grow and cause various forms of disease (Latge 1999). Most forms of the disease manifest in the lung and upper respiratory tract, which corresponds to the entry pathway of the conidia (Dagenais and Keller, 2009). There is, however, also infection of other organs such as liver, kidney, skin and brain which presumably might be due to hematogenous spread (dissemination) of the fungus on the basis of primary lung focus. However, there are also cases, particularly in cerebral aspergillosis, in which no pulmonary outbreak can be found (Denning 1998). In this part of study, the dissemination of pulmonary *A. fumigatus* infection to other organs was investigated in mice. So far, for dissemination of *Aspergillus* infection, only intravenous infection of mice has been reported but no adequate model of disseminated aspergillosis has been described after starting the pulmonary infection by intranasal infection. To investigate the changes in other organs in context of dissemination, sublethal doses of infection were used to avoid the lethal manifestation of the lung which generally occurs within 3-5 days of using lethal doses of conidia intranasally.

#### 3.2.1 Dissemination of *A. fumigatus* infection in immune suppressed mice

Mice were immune suppressed by cortisone acetate and $10^5$ conidia are routinely used in cortico-steroid mice model (Schöbel et al., 2010). Hence, to induce sub lethal infection of *A. fumigatus*, in order to follow the infection for longer time, cortisone acetate treated mice were infected intranasally with $10^4$ conidia. Three mice infected with *A. fumigatus* conidia were sacrificed per day and PBS injected mice served as negative control. Mice were examined at five different time points on days 1, 3, 5, 10 and 15 post-infection (p.i) to measure the fungal burden in lungs and other organs. The fungal burden was determined by calculating the fungal DNA among the total DNA isolated from each organ.

In immune suppressed mice, the pulmonary infection increased from day 1 to day 5 and subsequently showed a decrease in fungal burden of infected lungs on day 10 and day 15 (Fig 28). Furthermore, the other organs including kidneys, liver, brain, spleen were analyzed

for the presence of fungus. There was presence of low fungal DNA in kidneys and spleen in 2 mice on day 1 and day 3 p.i but no fungus was found in these organs at later time points (data not included). Furthermore, brain of infected mice also showed the presence of fungal DNA on days 3, 5 and 10 but not on day 1 and day 15 p.i (data not shown). By histopathological analysis, the fungus could not be detected in any organs except lungs. Also, on day one, lungs showed no presence of fungal mycelia, however, the mycelia were clearly visible after day 3 up to day 15 p.i (Fig 29).

To determine whether the fungus present in lungs and other organs was viable, all organs were homogenized and plated for reisolation of fungus. The results showed that the fungus was viable in lungs of immune suppressed mice on each day of mice sacrifice (Fig 30), however, there was no viable fungus in other organs of the infected mice which were found positive during PCR analysis.

Mice immune suppressed with cortisone acetate and infected with *A. fumigatus* were compared to cortisone acetate treated mice with PBS, taken as negative control. PBS mice showed no macroscopic alterations and there was no presence of fungal DNA in lung.

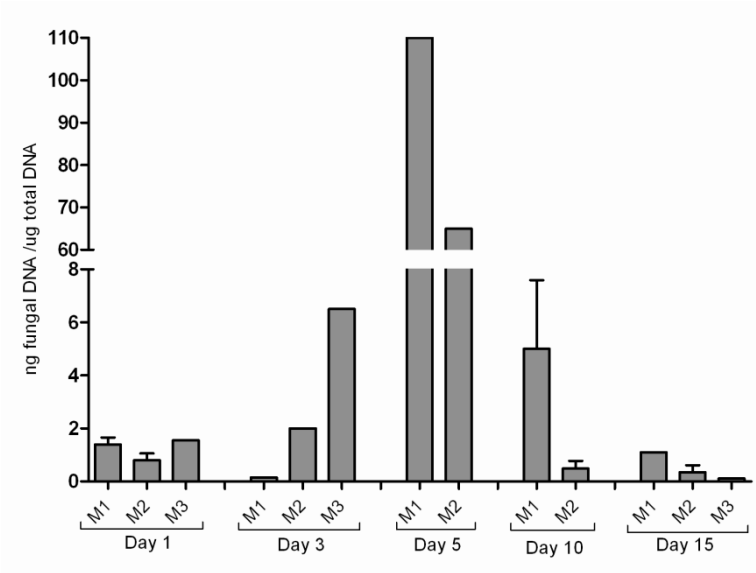



**Figure 28 Quantitative real-time PCR of fungal DNA from lungs of immune suppressed mice**
Mice were immune suppressed with cortisone acetate and sacrificed at day one, three, five, ten and fifteen after infection with *A. fumigatus* conidia. Mice infected with PBS were used as negative control and revealed no signal in PCR and is, therefore, omitted from the graph. The bars represent the amount of fungal DNA per microgram of total DNA isolated from the infected lungs with standard deviations from three data points for each individual animal. M1-M3 represent mouse number 1-3.

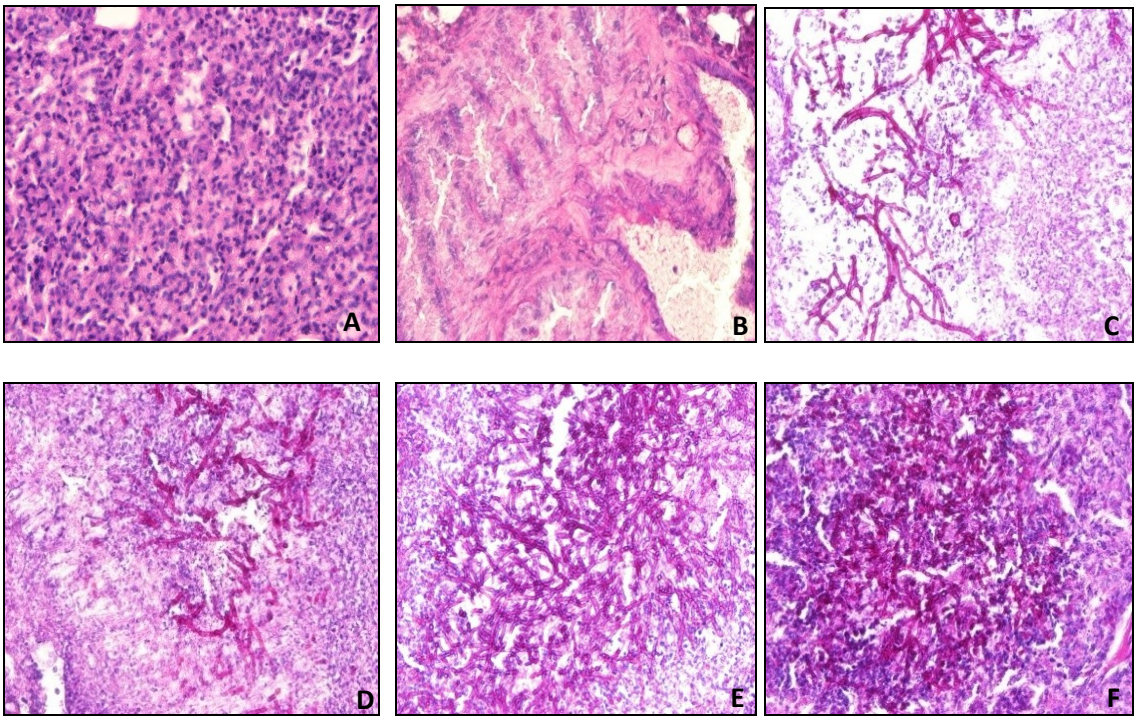



**Figure 29** The fungus was detected by PAS staining **A)** PBS injected mice as control. Infection with $10^4$ conidia after **B)** day 1, **C)** day 3, **D)** day 5, **E)** day 10 and **F)** day 15 post infection.

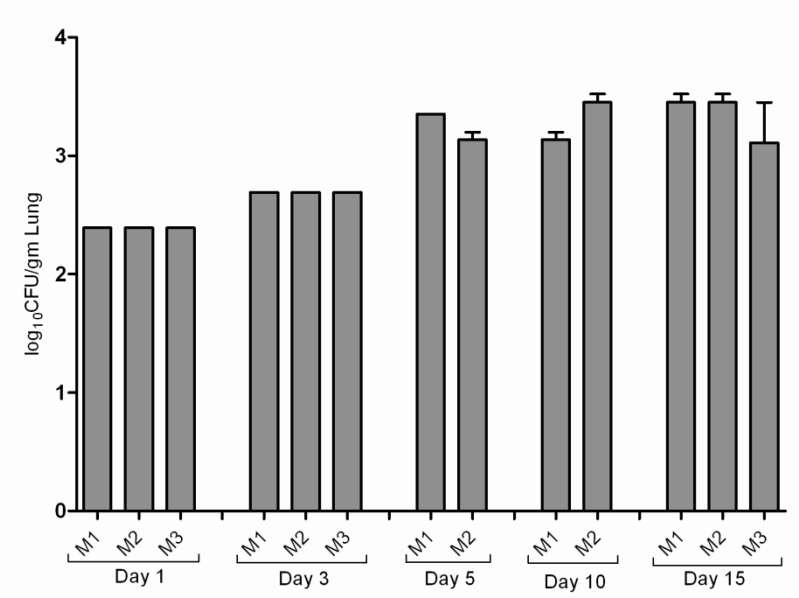



**Figure 30: Determination of viability of fungus in infected lungs of immune suppressed mice**

The fungus was reisolated on yeast malt agar plates and three replicates were taken for each lung. The mice were sacrificed on days one, three, five, ten and fifteen after infection with *A. fumigatus* conidia. The bars represent log $_{10}$ CFU per gram of lung tissue with standard deviations from three data points for each individual animal. M1-M3 represent mouse number 1-3.

### 3.2.2 Dissemination of *A. fumigatus* infection in immune competent mice

Next, immune competent mice were investigated for dissemination of infection after pulmonary infection and a sub lethal dose of $10^6$ spores was administered per mouse. The lungs of infected mice were aseptically removed on days 1, 3, 5, 10 and 15 after infection and analyzed for fungal burden. Three mice infected with *A. fumigatus* conidia were sacrificed per day and mice with PBS were used as negative control. The results showed that the fungal DNA was present in mice lung tissues on day one in all three mice which were sacrificed. One out of three mice sacrificed on day 3 showed the presence of fungal DNA, while as on day 5, two out of three mice showed the presence of fungal DNA in lungs (Fig 31). However, there was no presence of fungal DNA on days ten and fifteen p.i. The presence of fungal DNA was monitored in other organs but there were no traces of fungal DNA detected.

Since presence of fungal DNA does not relate to the viability of fungus, the fungus was reisolated on yeast malt agar plates. The fungus was reisolated on day one from one mouse out of three but there was no presence of fungal colonies on agar plates in immune competent lungs of other infected mice (data not shown). Negative control mice with PBS showed no presence of fungal DNA in lungs.

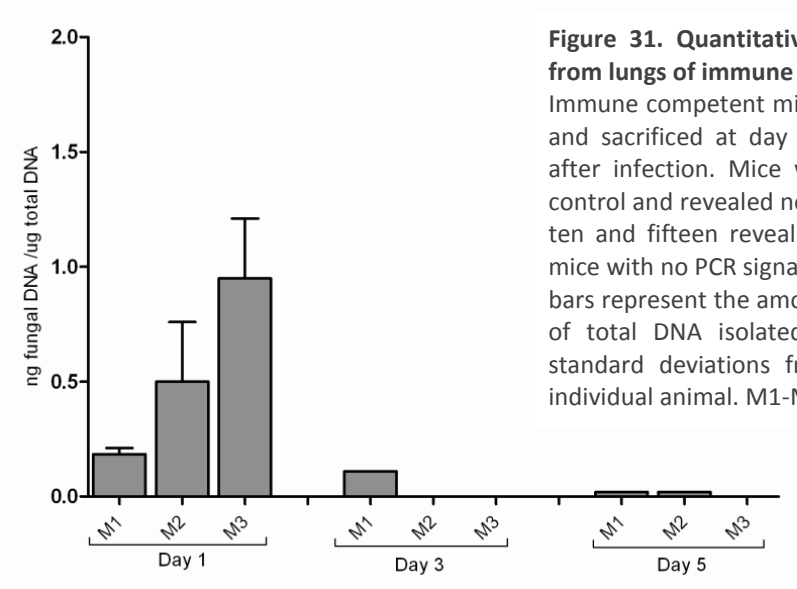



**Figure 31. Quantitative real-time PCR of fungal DNA from lungs of immune competent mice**
Immune competent mice were infected with $10^6$ conidia and sacrificed at day one, three, five, ten and fifteen after infection. Mice with PBS were used as negative control and revealed no signal in PCR. Also, mice on days ten and fifteen revealed no PCR signal and, therefore, mice with no PCR signal are omitted from the graph . The bars represent the amount of fungal DNA per microgram of total DNA isolated from the infected lungs with standard deviations from three data points for each individual animal. M1-M3 represent mouse number 1-3.

### 3.3 *In ovo* molecular Imaging of chick embryos and *in vitro* real-time molecular interactions as tools to characterize radiotracers for *Aspergillus fumigatus* infection

Several diagnostic strategies are currently used for detection of pulmonary infections caused by pathogenic fungi (Hope et al., 2005), the standard method among them being computed tomography or CT scan which nowadays is combined with positron emission tomography or PET scan (PET/CT). PET uses radiotracers and several attempts have been made for the development of radiotracers for the diagnosis of fungal infections (Ruchel et al., 2001; Lupetti et al 2005; Yang et al., 2009; Petrik et al., 2010; Petrik et al., 2014). However, detailed characterization and development of novel diagnostic tracers are carried out by various *in vitro* assays and further depends on the availability of suitable *in vivo* models. Mice have been used as golden standard model; however, their usage is limited due to ethical issues, time as well as resources needed for their handling. Further, these models require specialized skills and facilities and bear high cost. Due to these limitations, there is a need for more alternatives and chicken egg model can directly overcome these drawbacks. The purpose of this study was to demonstrate *in vivo* PET/CT imaging of chick embryos infected with *A. fumigatus* and *in vitro* real-time monitoring of tracer uptake by fungus using Ligand Tracer technology as tools to screen radiotracers for *A. fumigatus* infections. Ligand tracer technology was optimized to track the uptake of tracer by fungus grown on A549 cells. PET/CT imaging of *A. fumigatus* infection was demonstrated on the chorioallantoic membrane (CAM) of embryonated chicken eggs and further analyzed for the presence of fungal burden on CAM using desferri-triacetylfusarinine C (TAFC) labeled with Gallium-68. $^{68}$Ga-TAFC is an iron capturing siderophore and has previously been used for imaging *A. fumigatus* infection in rats (Petrick et al., 2010). Both the methods combined together could prove useful to speed up the screening of novel radiotracers for diagnosis of *A. fumigatus* infection in host.

### 3.3.1 *In vitro* characterization of radiotracer using Ligand Tracer® device

#### 3.3.1.1 Establishment of Ligand Tracer® device for *in vitro* characterization of radiotracer to follow *A. fumigatus* infection using $^{68}$Ga-TAFC

To characterize the uptake of radiotracer for *A. fumigatus* infection *in vitro*, LigandTracer device (white) was used. Ligand Tracer® white is preliminary applied for the examination of different cell lines, particularly suited for real time monitoring of cell surface receptors but has never been used for infection experiments. In this study, the instrument was evaluated to track the radiotracer uptake by *A. fumigatus* infected epithelial cells (A549 cell line) in order to establish protocol for characterization of new fungal tracers. Therefore, with the help of LigandTracer device, real time measurements of tracer ($^{68}$Ga-TAFC) uptake were done.

For establishment of system for infection processes, different measurement conditions were evaluated.

**Measurement conditions:**

Growth of fungus: In first experiments, the fungus was grown on a thin layer of different growth media like potato dextrose media, aspergillus minimal media and Sabouraud dextrose media, and monitored for radiotracer uptake. Due to complexity of media, the results could not be analyzed.

*A. fumigatus* was then grown on layer of lung epithelial cells (A549 cell line). The conidia attach to the cells and subsequently attach to the petri dish and grow into mycelia. A549 cells without *A. fumigatus* were used as control.

Temperature: A549 cell line and *A. fumigatus* conidia were grown at 37 °C in humidified temperature of 5% $CO_2$. In order to have the same temperature during measurement, the temperature of the system was increased from base settings of +25 °C to +37 °C and due to evaporation of solution at higher temperatures, the volume of tracer solution was increased from 1-2ml as recommended by the producer to 4 ml.

Forerun: To reach the temperature of +37°C, the system was pre-run for about 10 minutes especially during the first experiment of the day. During this time, the temperature can be adjusted in the machine and the cells can adapt to the differing conditions in the

measurement system. After a forerun of 10 min, the tracer was added and the data was collected.

Cell culture medium: For the first investigations, $^{68}$Gallium-TAFC was diluted in 1 x PBS buffer and added to cells. After a measurement time of about 40 minutes at +37 ˚C, an intense decrease of the curve was seen, which can be due to insufficient availability of nutrients in PBS during long measurement process (>30 min). This observation led to the decision that in all following experiments, $^{68}$Gallium-TAFC was diluted in RPMI medium without any additional supplements. After this adaptation, the data could be collected for more than one hour.

Infection dosage: In the first experiments, the cells were infected with MOI of 10 and the tracer uptake was measured. Due to dense growth of mycelia, there was partial detachment of culture on plates due to which the analysis of result was not possible. The reason for this was the high infection dosage and mechanic exposure due to the rotation of the petri dish in the machine. Therefore, the cells were infected with MOI of 5 for 12 hours. Detachment of the cells from the petri dish could not be observed under these conditions.

#### 3.3.1.2 *A. fumigatus* infected cells show higher uptake of $^{68}$Ga-TAFC than uninfected cells and the mutants lacking tracer uptake activity

Gallium-68 labeled TAFC ($^{68}$Ga-TAFC) uptake was measured after 12h of co-incubation of cells with *A. fumigatus* spores at multiplicity of infection of 5. *A. fumigatus* (wild type) co-incubated with A549 cells for 12 h showed high uptake of $^{68}$Ga-TAFC during the time of measurement. After washing the plate with PBS as well as replacing the remaining radioactive tracer solution with clean PBS, radioactivity could still be detected which was attached to the infected cell culture (Fig 32 A). This indicated that the tracer was retained by fungal mycelia cells. A549 cells alone without *A. fumigatus* did not show any uptake or retention of tracer during a run of 90 min (Fig 32 B).

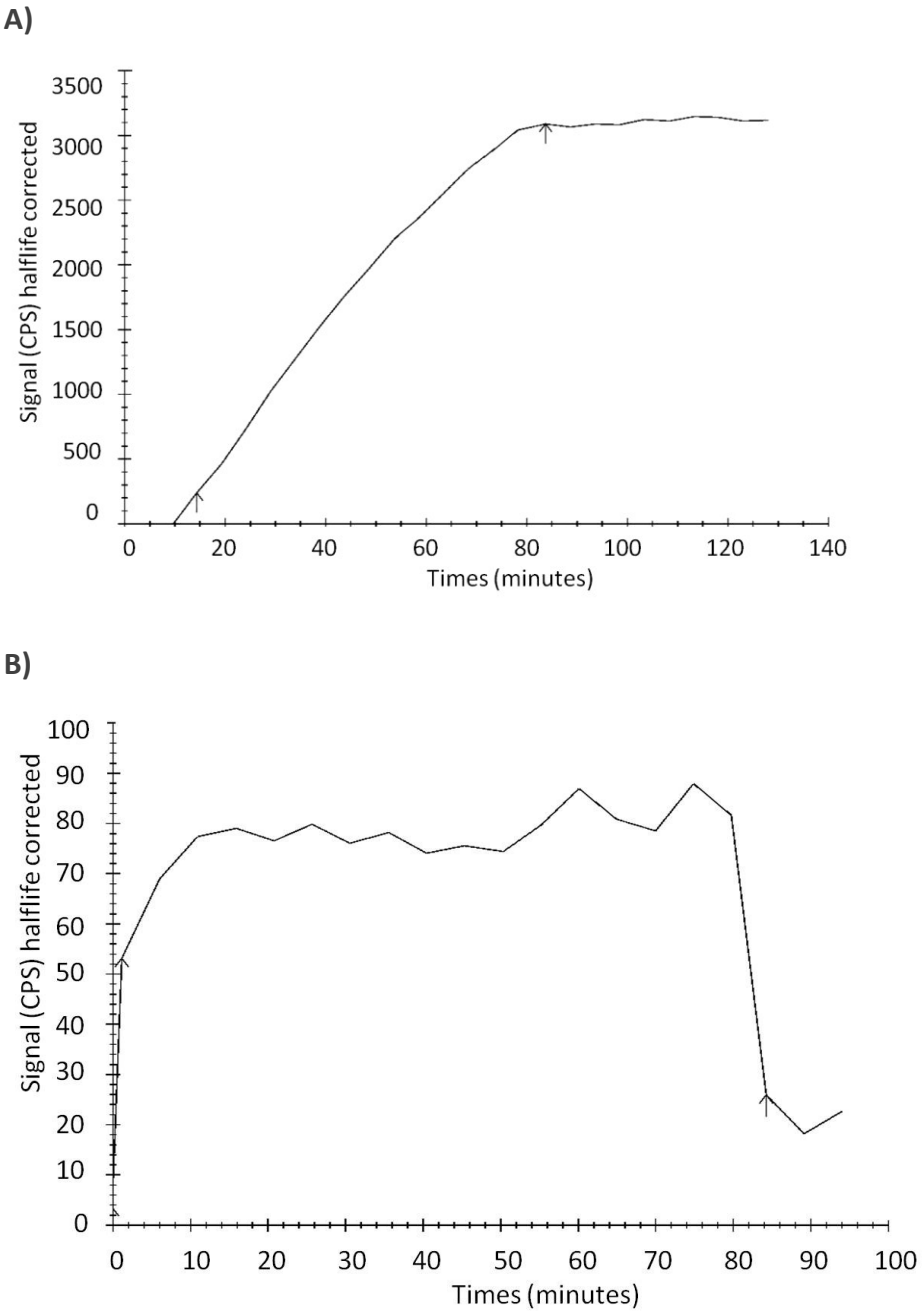


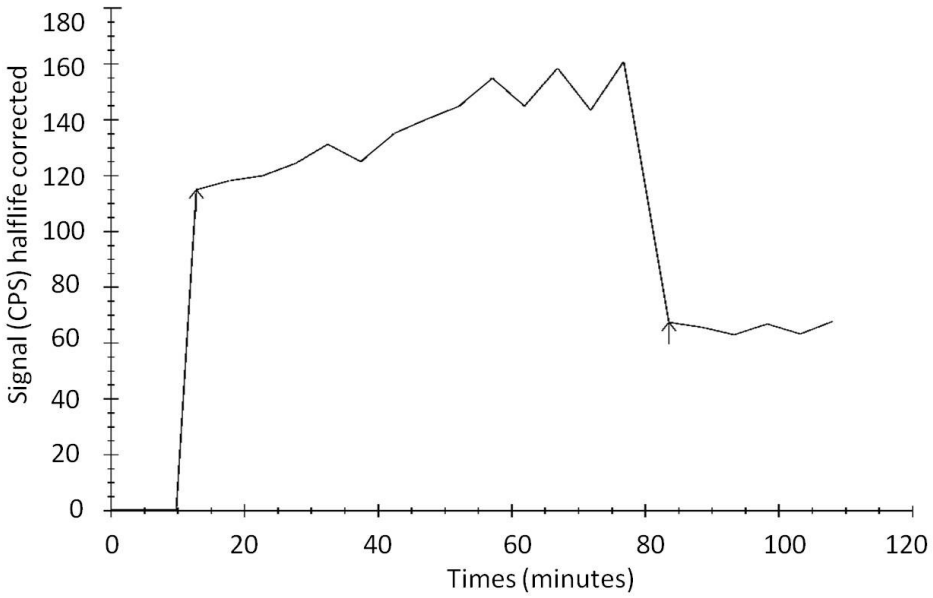

**Figure 32. Uptake of $^{68}$Ga-TAFC by *A. fumigatus* infected A549 cells using Ligandtracer device**

A549 cells were infected with *A. fumigatus* and measurement was taken 12 hours post infection. A549 cells without *A. fumigatus* were used as control measurements. Data corrected for the half life of $^{68}$Ga. **A)** Uptake of $^{68}$Ga-TAFC by *A. fumigatus* wild type infected A549 cells. **B)** Control A549 cell line without *A. fumigatus.* The graph is representative of two independent experiments.

The transcription factor HapX in *A. fumigatus* activates the iron uptake by siderophores during iron starvation conditions (Schrettl et al., 2010) and hence, *hapX* mutant is unable to take up the siderophores. So, *hapx* mutant was tested for real time measurement of

radiotracer due to the lack of siderophore uptake property. The *hapX* mutant, like control A549 cells, did not show any uptake of $^{68}$Ga-TAFC tracer (Fig 33 A). Taken together, A549 cells infected with *A. fumigatus* spores showed higher uptake of $^{68}$Ga-TAFC tracer compared with uninfected cells and *hapX* siderophore mutant, for example, at time point of 80 mins, cells infected with *A. fumigatus* showed signal of 3000 counts per second (CPS) (half life corrected) while as uninfected cells and cells infected with *hapX* mutant at same time point showed CPS value of about 80 and 150 respectively (Fig 33 B).

**A)**




**B)**

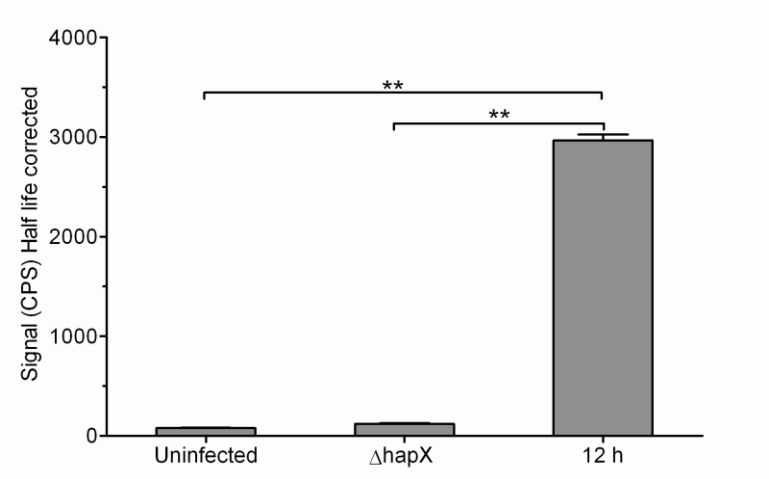



**Figure 33. Uptake of $^{68}$Ga-TAFC by hapX mutant using Ligandtracer device**

A549 cells were infected with *hapX* mutant spores and incubated for 12 hours. **A)** Upatke of tracer by Δhapx mutant lacking siderophore uptake property. The graph is representative of two independent experiments. **B)** Comparison of radiotracer uptake by cells with or without *A. fumigatus* and with hapX mutant. Values represent the mean+SD of three experiments. (*, $P \leq 0.05$; **, $P \leq 0.01$, calculated by Mann-Whitney U test).

### *3.3.2 In ovo* characterization of radiotracer using embryonated chicken eggs as model.

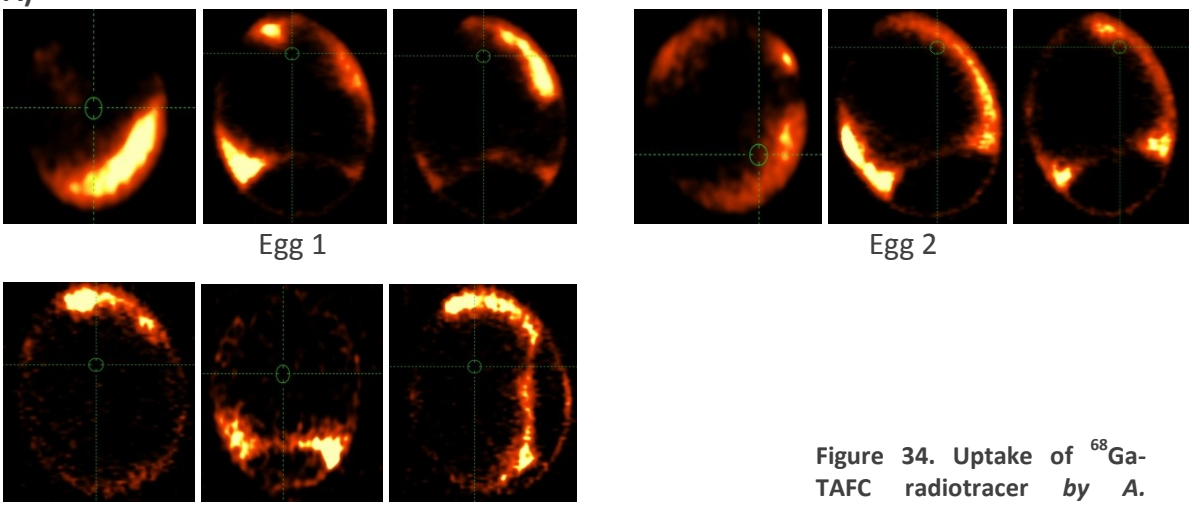
#### 3.3.2.1 Establishment of chicken eggs for *in ovo* characterization of radiotracer to follow *A. fumigatus* infection using $^{68}$Ga-TAFC

*In vitro* characterization of radiotracer is generally followed by *in vivo* characterization using different animal models. Here, embryonated chicken eggs were used as an *in ovo* test system for the evaluation of newly developed tracers to track the infections caused by *A. fumigatus*. Embryonated chick eggs have been used as an alternative model system for fungal pathogens such as *A. fumigatus* (Jacobsen et al., 2010) and *Candida albicans* (Jacobsen et al., 2011). However, the molecular imaging by PET and CT in combination with embryonated chicken eggs as an alternative *A. fumigatus* infection model has not been reported so far. Based on the study with infection models, an infection dose of $10^4$ per egg was chosen as this dosage renders a sub lethal effect on chicken eggs. The infection was done on developmental day 10 via chorioallantoic membrane (CAM). The imaging was carried on at developmental day 13 (3 days p.i.) because infection dose of $10^4$ resulted in death of embryos within 4 days.

#### 3.3.2.2 PET/CT imaging of *A. fumigatus* infected chicken embryos

From a group of 10 infected chicken embryos, three were randomly selected for imaging. For getting artifact free images, the eggs were anesthetized using vaporized isoflurane (Heidrich et al., 2011). The eggs were subjected to CT scan first followed by PET measurements. In normal non-infected embryonated chicken eggs, $^{68}$Ga-TAFC showed rapid distribution over the CAM but no hotspots of tracer uptake could be visualized (data not shown). Comparatively, the infected embryonated chicken eggs showed tracer uptake at various spots designated as regions of interest (ROIs) (Fig 34 A). In infected egg 1, there were two hot spots of tracer uptake observed with %ID/g values of 13.2 and 9.6, while in egg 2, three hot spots of tracer uptake were visualized having the values of %ID/g as 12.93, 7.33 and 6.4. Additionally, egg 3 showed only one hot spot with %ID/g value of 4.12% (Fig 34 B). %ID/g values give the percentage of infected tracer dose per gram of tissue.

**A)**




**B)**

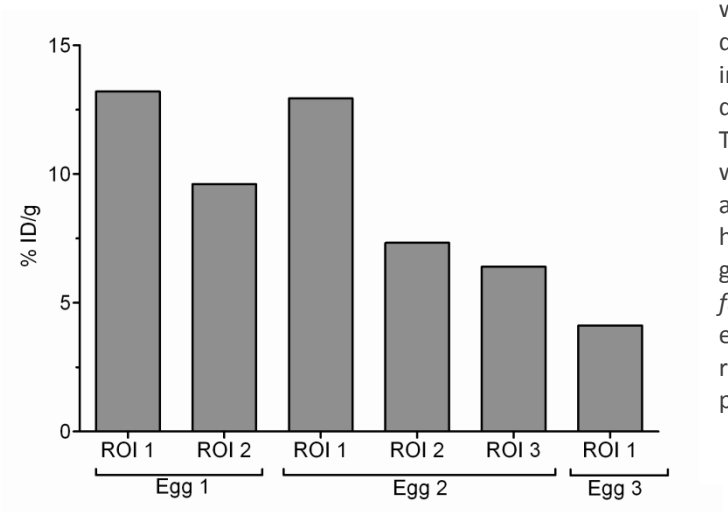
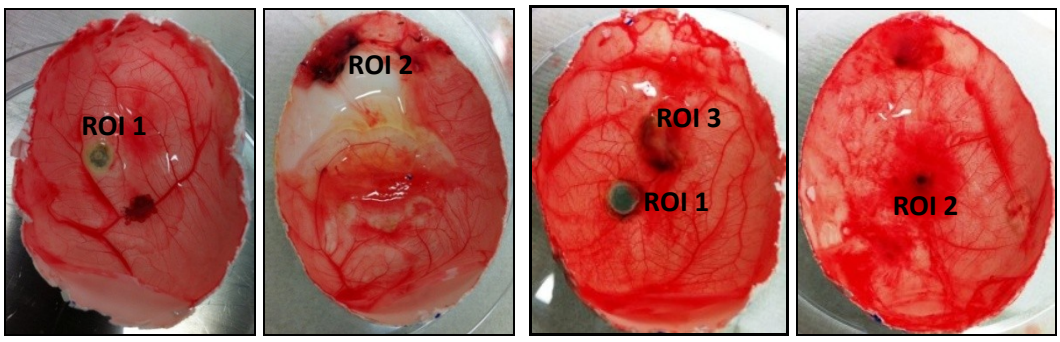


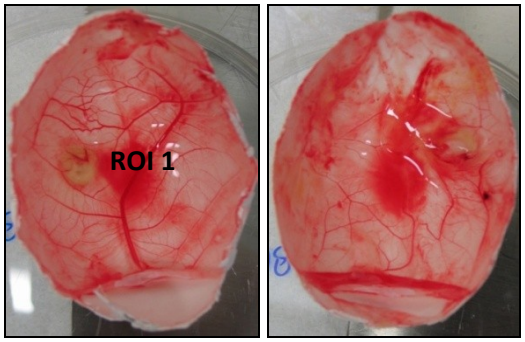

**Figure 34. Uptake of $^{68}$Ga-TAFC radiotracer *by A. fumigatus* infected chicken eggs by PET imaging.**
Chick embryos were infected with *A. fumigatus* on developmental day 10. PET imaging was done after three days of infection using $^{68}$Ga-TAFC. Chick embryos with PBS were used as negative control and revealed no PET signal, hence, are omitted from graph. **A)** PET images of *A. fumigatus* infected chick embryos. **B)** The graph represents the infected dose per gram of tissue.

### 3.3.2.3 Correlation of PET signals with fungal burden in infected chick embryos

After imaging of *A. fumigatus* infection in embryonated chicken eggs, the presence of fungus on CAM and its distribution was macroscopically observed. The eggs were kept on ice for one hour and opened aseptically. Several visible spots of gray green fungus were found on CAM of infected chick embryos which were aseptically removed and further processed for fungal load (Fig 35).




Egg 1 Egg 2




Egg 3

**Figure 35. Macroscopic analysis of *A. fumigatus* infected chick embryos**.
Presence of fungal colonies on CAM of chick embryos. The fungal spots on each egg were aseptically removed; designated as regions of interest (ROIs).

The fungal load at ROIs was quantified by calculating the fungal DNA among the total DNA isolated from each spot. In Egg 1, four spots of fungus were isolated and two out of four spots showed the presence of fungal DNA while as in Egg 2, three out of four fungal spots showed the presence of fungal DNA. Furthermore, three spots out of four showed the presence of fungal DNA in egg 3 (Fig 36). To check the viability of fungus on these regions of interest, the fungus was reisolated on yeast malt agar plates. All ROIs showed the presence of viable fungus which was in accordance with PCR quantification (Fig 37). The embryos injected with PBS showed no alterations on CAM and there was no presence of fungal DNA. Taken together, embryonated chicken eggs can be used as *in vivo* model to test the novel radiotracers for diagnosis of *Aspergillus* infections which can directly lead to the reduction of animal models.

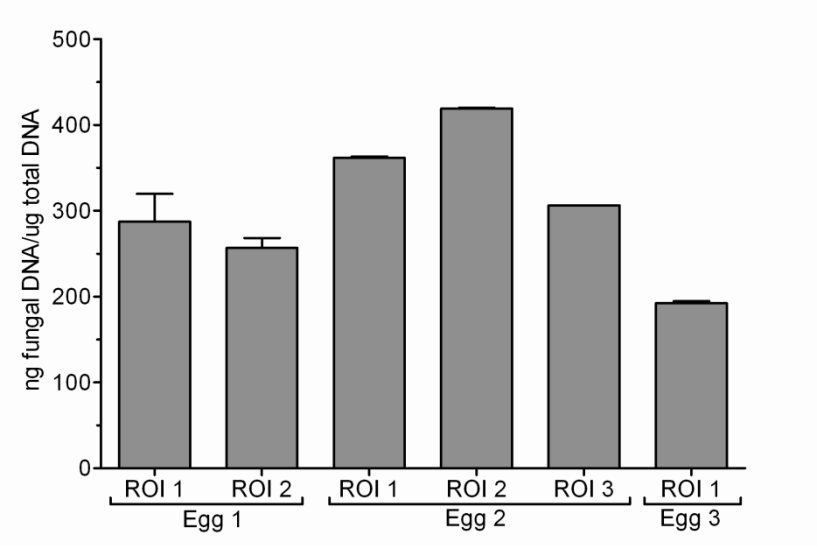



**Figure 36. Quantitative real-time PCR of fungal DNA from CAM of embryonated chicken eggs**

Embryos were infected with $10^4$ dose of *A. fumigatus* conidia and sacrificed on day three after infection. As negative control, PBS was injected via CAM and showed no presence of fungus and is, hence omitted from the graph. The bars represent the amount of fungal DNA per microgram of total DNA isolated from regions of interests (ROIs) on CAM with standard deviations from three data points.

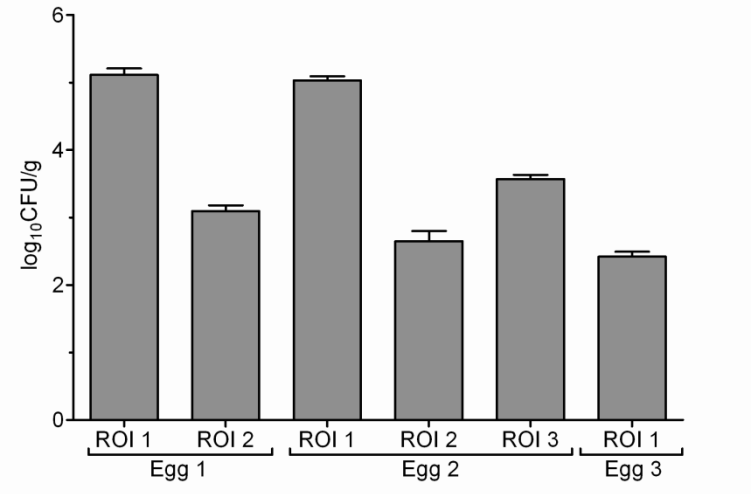



**Figure 37. Determination of viability of fungus in infected CAM of embryonated chicken eggs**

The viability of fungus was checked by reisolating the fungus on yeast malt agar plates. Three replicates were taken and the embryonated eggs were sacrificed on days three after infection with *A. fumigatus* conidia. The graph represent $\log_{10}$ CFU per gram of CAM (mean + SD)

# 4 Discussion

## 4.1 Melanin impacts survival of *A. fumigatus* conidia in lung epithelial cells

Many pathogenic microorganisms enter host cells and often use this originally hostile environment as a niche to evade the host immune response. Upon inhalation, *A. fumigatus* conidia reach the pulmonary alveoli where they interact with alveolar epithelial cells (Brakhage et al., 2010; Latge, 1999). The epithelial cell layer represents an initial point of contact between fungus and host. Furthermore, the initial stages of interaction between host and *A. fumigatus* are important in establishment of infection caused by fungus, however, little research has addressed the understanding of this interaction between epithelial cells and *A. fumigatus* conidia (Osherov, 2012). Previously, it was shown that *A. fumigatus* conidia are internalized by epithelial cells (Amitani and Kawanami, 2009; Wasylnka and Moore, 2002). In this study, the uptake of conidia, their internal processing and their potential persistence in context with alveolar epithelial cells was analyzed. Manipulation of host cell death provides a survival advantage to many pathogens. *A. fumigatus* has been shown to exert both pro- and anti-apoptotic properties depending on its morphological forms. For example, hyphae induce apoptosis in host cells via secretion of the secondary metabolite gliotoxin (Pardo et al 2006; Stanzani et al 2005) while conidia were shown to inhibit apoposis in epithelial cells (Berkova et al., 2006, Femenia et al., 2009) and macrophages (Volling et al., 2007). Here, it was demonstrated that DHN melanin, which exerts cytoprotective effects on macrophage apoptosis (Volling et al., 2011), is responsible for inhibition of apoptosis in lung epithelial cells. For the first time, here, it was shown that the internalization of *A. fumigatus* conidia by epithelial cells depends on the conidial pigment DHN melanin, which was reported to protect the conidia from macrophage recognition during phagocytosis. However, unlike macrophages, it was shown here that DHN melanin is a critical factor for uptake of conidia by epithelial cells.

### 4.1.1 *A. fumigatus* conidia reside and survive in lung epithelial cells

The lung alveoli is mostly the initial site to establish infection caused by *A. fumigatus* (Hope et al., 2007). Given that the first site of contact of *A. fumigatus* conidia with its host is the respiratory epithelial surface, only few studies have analyzed this interaction. Once the

infectious conidia are inhaled and deposited in lungs, they encounter various pulmonary defenses such as respiratory mucous, soluble innate immune effector molecules and pulmonary phagocytic cells (Hope, 2007). The lung alveolus is mostly associated with the pathogenesis of invasive pulmonary aspergillosis due to the fact that the conidia are airborne and the gas exchange involves the alveolar capillary barrier (Hope, 2009). A significant proportion of inhaled conidia contact epithelial cells before coming in contact with alveolar macrophages and therefore, play an important role in host defenses. In immunocompromised patients, the inhaled conidia may germinate and develop into fatal infection, known as invasive aspergillosis (Denning DW, 1998). Moreover, in patients at risk of invasive aspergillosis, there is a frequent damage of airway epithelium by radiations, chemotherapy, or by viral and bacterial infections (Cordonnier C et al., 1996). *A. fumigatus* conidia and hyphae are shown to induce endocytosis by type II pneumocytes and adhere rapidly to epithelial cells (DeHart et al., 1997; Paris et al., 1997). Once these conidia are internalized by epithelial cells, germination of conidia is delayed. The engulfed conidia co-localise with acidified phagosomes and are killed, however, some conidia persist and undergo germination without affecting the viability of cells (Wasylnka and Moore, 2002). Therefore, it is reasonable to assume that the uptake of *A. fumigatus* conidia by pulmonary epithelial cells represents a mechanism of immune evasion allowing conidia to persist in the hostile environment of the lung. If this possibility was true, it is also conceivable that invasive aspergillosis starts from intracellular conidia as soon as the immune system is impaired. Respiratory pathogens such as *Haemophilus influenzea* invade epithelial cells to facilitate the migration while as *Mycobaterium leprae* replicate inside epithelial cells (Bermudez and Goodman, 1996). Other pathogens such as *Salmonella* spp., *Yersenia* spp. use epithelial cells as niche to survive within the host by avoiding the host defences (Miller and Falkow, 1988; Finlay and Falkow, 1988). In the present study, the presence of non-germinated conidia inside epithelial cells after 24 h of co-incubation, by using confocal microscopy, suggests the possibility of persistence of conidia inside these cells which is also possessed by various bacterial pathogens in asociation with epithelial cells.

Pathogens use a variety of novel mechanisms that balance breaching the epithelial cell layer with the maintainence of epithelium to allow the colonization and adherence of invading pathogens (Kim, M., et al, 2010). On interaction of *A. fumigatus* with epithelial cells, the cells

remained intact and some internalized conidia started forming mycelial fragments outside the cells. Here, by using the uracil-auxotrophic strain CEA17, the fate of these intracellular non-germinated conidia was followed and the interference of germinating mycelia was avoided. When supplemented with uracil/uridine, these conidia showed wild-type phenotype. Analyses using microscopy and survival assays showed that *A. fumigatus* conidia were able to reside and survive inside the cells for up to 24 h. Taken together, *A. fumigatus* conidia are able to reside inside epithelial cells forming a niche inside these cells and are able to survive within them.

### 4.1.2 DHN melanin increases the internalization of conidia by epithelial cells

The characteristic grey-green colour of *A. fumigatus* conidia derives from DHN melanin, which was found not only to protect the fungus against various stresses such as ROI but also to modulate the immune response of the host (Chai et al., 2011; Jahn et al., 1997; Jahn et al., 2002). Recently, DHN melanin was shown to interfere with the host endocytotic pathway in both macrophages and neutrophils (Heinekamp et al., 2012; Thywissen et al., 2011). Resting conidia with intact layers of melanin and hydrophobic rodlet layer on cell wall renders the conidia inert to host immune system (Aimanianda, 2009). The binding and ingestion of resting conidia induce very little inflammatory response (Gersuk, G.M., et al., 2006; Hohl, T.M et al., 2005; Steele, C., et al., 2005), and optimal $CD4^+$ T-cell responses appear to occur only in response to live conidia (Rivera, A. et al., 2005). Thus, one of the critical factors in host-fungal interplay is the morphological state of *Aspergillus*. The mechanism associated with adhesion and internalization of *A. fumigatus* conidia by epithelial cells is unclear. Hence, here, the influence of DHN melanin was analyzed by generating a pigmentless uracil-auxotrophic *A. fumigatus* mutant which did not germinate within cells. This experimental setup allowed to compare the long-term interaction of melanised and non-melanised conidia with epithelial cells. Such experiments were more difficult with uracil-prototrophic conidia, because many of them germinated and produced hyphae, which overgrew the cell layer. As an indication of validity, both uracil-prototrophic and uracil-auxotrophic wild-type and *pksP* mutant strains were exemplarily analysed.

Unlike professional phagocytes, the presence of melanin enhanced the uptake of *A. fumigatus* conidia by epithelial cells when compared with *pksP* mutant conidia. Previously, it

was reported that the lack of DHN melanin on the surface of conidia makes the underlying carbohydrates of the cell wall more accessible to sensing by receptors like dectin-1, which could explain the better uptake of pigmentless *pksP* mutant conidia compared with wild-type conidia by macrophages (Brown et al., 2002; Luther et al., 2007). The unexpected higher uptake of melanised conidia by epithelial cells might be due to different receptors on the surface of these cell types, which could be specific for melanin in epithelial cells. It is even conceivable that the increased uptake of wild-type conidia reflects an immune evasion mechanism as conidia can survive intracellularly and thus find a niche inside cells. Alternatively, a defect in melanin biosynthesis in *A. fumigatus* was suggested to contribute to the loss of adherence properties of conidia because DHN melanin is a hydrophobic molecule with a negative charge (Pihet et al., 2009), which in case of epithelial cells can be a cause of lower uptake of *pksP* mutant conidia compared with wild-type conidia. However, it would be difficult to understand why this does not account for macrophages. Furthermore, in line with the argumentation that in particular melanised conidia are more efficiently taken up is the observation that inactivated resting conidia and not swollen conidia, which are supposed to have lost most of their melanin layer, were recognized and uptaken by respiratory epithelial cells (Beisswenger et al., 2012). Recently, gliotoxin, produced by fungal hyphae is shown to promote the internalization of *A. fumigatus* conidia in lung epithelial cells (Jia X et al., 2014).

Adherence to host constituents is regarded as one of the important virulence factors in various pathogens including *A. fumigatus* but little is known about the molecular mechanisms underlying the adherence of *A. fumigatus* to host constituents (Sheppard, DC, 2011). Various fungal factors such as rodlet layer (Aimanianda V et al., 2009; Thau N et al., 1994), galactosaminogalactan (Gravelat et al., 2010; Gravelat et al., 2013) and various proteins (Gil et al., 1996; Penalver et al., 1996; Tronchin et al., 1997; Banerjee et al., 1998) are suggested to mediate the adherence of *A. fumigatus* to host cells. Melanin, as shown here, contributes to the factors invloved in adherence of *A. fumigatus* conidia to host cells such as alveolar epithelial cells.

From host point of view, recognition of invading pathogens and intracellular processing of engulfed organisms, such as, induction of host cell death or apoptosis and phagolysosomal

acidification contribute to immune defences against invading microorganisms. However, to survive within the host, pathogens inhibit or overcome the immune attacks from phagocytic cells of the host. Prevention of host cell death and phagosomal maturation are common mechanisms used by various pathogens to evade the host immune system (Flannagan et al., 2009; Haas, 2007). Intracellular pathogens such as *Mycobacterium tuberculosis* and *Listeria monocytogenes* have evolved different mechanisms to escape the phagosomal death (Fratti et al, 2001; van der Wel et al., 2007, Singh et al., 2008). *Histoplasma capsulatum* inhibits the acidification of macrophage phagolysosomes containing the yeast to evade the host immune defense (Eissenberg et al., 1993; Newman et al., 2006). In professional phagocytes, *A. fumigatus* is able to prevent full acidification of phagolysosomal compartments which is dependent on its ability to produce DHN-melanin (Thywissen et al., 2011). In contrast to previous results (Wasylnka and Moore, 2003), no acidification of phagolysosomes containing wild-type conidia during 24 h of co-incubation was observed in alveolar epithelial cells which is in accordance to previous results where it was found that *A. fumigatus* is able to prevent phagolysosomal acidification in professional phagocytes (Slesiona et al., 2012) as well as in non-professional phagocytes indicating that inhibition of phagosomal biogenesis is a ubiquitous mechanism (*personal communication* Andreas Thywissen). However, in contrast to professional phagocytes, the conidia containing compartment of epithelial cells undergoes drastic morphological changes leading to a vacoular-like structure containing several conidia and additional intravacoular vesicles (Paris et al., 1997). If containing non-melanised conidia, those compartments still possessed acidic properties which indicated a possible antimicrobial capacity in contrast to wild type conidia containing compartments (*personal communication* Andreas Thywissen).

### *4.1.3 A. fumigatus* conidia repress apoptosis in alveolar epithelial cells

Alveolar epithelial cells are an important part of innate immune system with role in defense against *A. fumigatus* infections. Epithelial cells, like other phagocytic cells, produce proinflammatory cytokines and chemokines (Balloy et al., 2008; Bellanger et al, 2009) and contribute to effective recruitment of other immune cells (Mukaida, N., 2000, Pease and Sabroe 2002). In immunocompromised patient, such as CGD patients or treatment with coticoteroids, there is a higher risk of developing invasive aspergillosis (Denning DW 1998;

Gallien et al., 2008). Furthermore, the frequent damage of epithelial layer by chemotherapy, radiations and by other infections such as those caused by bacteria and viruses, are at higher risk of developing invasive aspergillosis. Apoptosis or suicidal cell death is one of the mechanisms of innate immune response against invading pathogens and manipulation of apoptosis is one of the critical determinants in outcome and progression of infections caused by various pathogens. Pathogenic microorganisms can manipulate host cells by applying various strategies. Some pathogens evade the innate immune response by manipulating host cell apoptosis. For example, *Shigella flexneri, Chlamydia* and *Neisseria* inhibit apoptosis of epithelial cells to escape the host defence (Clark and Maurelli, 2007; Suzuki et al., 2005). Thus, inhibition of host cell death is an important strategy used by pathogens to cause infection in host (Rodrigues et al., 2012). *A. fumigatus* conidia were shown to suppress apoptosis in various cell types including type II pneumocytes (Berkova et al., 2006; Femenia et al., 2009; Volling et al., 2007). However, the study on type II pneumocytes only analysed a short-term interaction (6 h) because germination of conidia on epithelial cell cultures hindered long-term studies due to hyphal overgrowth of the cell cultures. In this study, the experimental setup using uracil-auxotrophic mutant strains allowed to study the long-term interaction and to elucidate in detail the factors important for the inhibitory effect of conidia on epithelial cells.

Apoptosis can be initiated either via immunological killing molecules such as TNF-α that induce death receptor signalling or via intrinsic stimuli such as ROI that induce the intrinsic mitochondrial apoptosis pathway. In context of this thesis, the impact of conidia on STS-induced (intrinsic pathway) and TNF-α/CHX-induced (extrinsic pathway) apoptosis in epithelial cells was analyzed. Here, it was found that *A. fumigatus* conidia are able to inhibit both apoptotic pathways in A549 lung epithelial cells even for longer periods as measured by flow cytometry and immunoblotting. The members of caspase family which are present as inactive pro-enzymes, become activated upon proteolytic cleavage during the process of apoptosis. Caspase-3 which is an executioner or effector caspase acts as a common link between two classical pathways, i.e., extrinsic and intrinsic apoptotic pathways. However, STS-induced apoptosis in epithelial cells did not reveal caspase-3 activation. This can be due to the fact that in epithelial cells, STS may be involving a caspase-independent pathway. The observed cytoprotection increased with the increase in contact of conidia with epithelial

cells which might be due to increased internalized conidia in epithelial cells. In immunocompetent individuals, *A. fumigatus* mediated apoptotic inhibition of epithelial cells is particular important as not all ingested conidia are killed by these cells due to the fact that these cells are not "real" phagocytes. Besides this, the anti-apoptotic effect of *A. fumigatus* conida might lead to reduction in killing of ingested conidia. The inhibition of apoptosis by ingested *A. fumigatus* conidia may provide a niche for the fungus to survive inside the host cells and breach the barrier once the immune system is lowered, for example in immunocompromised patients. Hence, *A. fumigatus* mediated repression of apoptosis of epithelial cells may contribute to the virulence of *A. fumigatus.*

### 4.1.4 DHN melanin is essential for inhibition of apoptosis in epithelial cells

The first structures of invading pathogens encountered by immune cells are the outer cell wall components. *A. fumigatus* resting conidia are covered by the proteinaceous hydrophobic rodlet layer that is released during swelling (Dague, E., et al 2008) and a DHN-melanin layer which forms the outer cell wall of conidia and immunologically render the fungal cellss inert to the inflammatory response of the host upon ingestion of conidia. These structures play an important role in manipulation of host immune responses. Previously, it was shown that conidia inhibit host cell apoptosis in macrophages and melanin was found to be a crucial factor for this inhibitory activity (Volling et al. 2011). In epithelial cells, also *A. flavus* but not *A. niger* and *A. nidulans* were found to inhibit apoptosis (Femenia et al., 2009). Furthermore, an unknown anti-apoptotic factor released by *A. fumigatus* conidia was suggested to be responsible for sustained survival of infected cells (Femenia et al., 2009). However, recent studies indicated that phagocytosis of conidia and thus physical contact is required for inhibition of apoptosis (Volling et al., 2011). Here, the effect of *A. fumigatus* melanised and non-melanised conidia, DHN melanin ghosts, and different types of DOPA melanin (natural and synthetic) on apoptosis of epithelial cells was analyzed. Unequivocally, it was shown that the melanins studied exert an inhibitory effect on TNF-α/CHX induced apoptosis of epithelial cells. Similarly, DOPA-melanin was previously shown to inhibit apoptosis in retinal epithelium (Seagle et al., 2006). The white *pksP* mutant lacking the polyketide synthase of melanin biosynthesis was not able to inhibit apoptosis of epithelial cells. Furthermore, non-melanised conidia are attenuated in virulence when tested in mouse

infection studies (Jahn et al., 1997; Tsai et al., 1998). Because both types of melanin, DOPA and DHN, inhibit apoptosis, it is likely that their common ability to scavenge ROI is responsible for their anti-apoptotic effect. Consistently, it is well known that generation of ROI promotes apoptotic cell death (Carmody and Cotter, 2001; Chandra et al., 2000; Jacobson, 1996). Additionally, melanin has been shown to neutralize potentially harmful unpaired electrons arising from ROS (Commoner et al 1954; de Cassia and Pombeiro-Sponchiado, 2005; Jacobson 2000; Liu and Nizet 2009) and also is able to detoxify antifungal drugs such as amphotericin B, which is often used for treatment of invasive fungal infections (Metcalf and Dockrell, 2007). Though melanised conidia are taken up more rapidly than non melanised conidia by alveolar epithelial cells, this is presumably another regulatory mechanism which is mediated by DHN-melanin to interfere with host immune response. As seen in macrophages (Volling et al., 2011), melanised conidia inhibit host cell apoptosis in epithelial cells as shown here. These effects observed in epithelial cells contribute to the pathogenecity of *A. fumigatus*.

### 4.2 Dissemination of *A. fumigatus* after pulmonary infection in mice

Fungal infections have been steadily increasing in recent decades as important causes of nosocomial infections. In certain groups of patients, these infections are now as big a threat as bacterial pathogens. A better understanding of the fungal pathogenesis is urgently required to develop rational approaches for early diagnosis and more effective therapies. In the second part of this study, the attempt was to look into the question whether *A. fumigatus* disseminated in a mouse model and whether it could be established, based on the pulmonary infection, as an animal model for the dissemination and reactivation of *A. fumigatus* infection. Due to poor therapeutic results and inadequate diagnostic possibilities for early detection of aspergillosis in humans, this animal model for the dissemination of infection could be of great importance for the possible improvement of diagnostic and therapeutic approaches. Since the fungal inoculum is important for determination of lethality in infection models (Dixon et al., 1989; Stephens-Romero et al., 2005), hence, a sub-lethal infection dose of *A. fumigatus was* administered intranasally to immunocompromised and immunocompetent mice. The sublethal doses were given so that animals could survive longer and therefore, analyzed for the dissemination of infection to other organs starting

from lungs. In context to this study, it was found that in immunocompromised mice, besides the presence of fungus in lungs, the fungal DNA and not viable fungus was present in brain. However, with this experimental set up, there is need of further establishment of animal models for disseminated aspergillosis after pulmonary infection. With immunocompetent mice model for dissemination of infection, the fungus was cleared within 24 hours and there was no presence of fungus in other organs.

### 4.2.1 Dissemination of *A. fumigatus* infection in immune suppressed mice

*A. fumigatus* is the main cause of invasive pulmonary aspergillosis (IPA), which is characterized by an invasive fungal growth in the lung tissue (Latge 1999) and immunocompromised persons are especially at a high risk of such fungal diseases. By an insufficient host immune response, these conidia are not cleared in a sufficient way (Schaffner et al., 1982). The conidia germinate and develop into mycelium in lungs which lead to necrosis and destruction of blood vessels (Stergiopoulou et al., 2007). In addition to the frequent pulmonary manifestation, infections of upper respiratory tract including sinuses, the eyes, heart, liver, kidney, skin, bone and brain are also described (Denning D.W 1998). The majority of patients with cerebral aspergillosis also show manifestations in other organs, suggesting a hematogenous dissemination which seems to be the penetration of fungus into blood vessels as can be regularly observed in mouse models of pulmonary infection (Ibrahim Granet et al., 2010).

In this study, the use of corticosteroid mouse model infected with a sub lethal dose of conidia helped in investigating the infection for longer time periods. Corticosteroids lead to impaired killing of microorganisms by phagocytes (Lewis and Kontoyiannis 2009). To mimic the clinical infectious process and also to cause pulmonary infection, the conidia were applied intransally to immune suppressed mice. Sub lethal doses of *A. fumigatus* infection caused an increase in fungal burden initially, which decreased later due to immune response, as in this immune suppressed model of infection, immune cells are still recruited to site of infection (Dixon et al. 1989; Brock et al. 2008). Furthermore, it should be noted that the effects of cortisone treatment on the immune system are only temporary and that the immune system regenerates towards the end of the infection experiments. Also, it was

demonstrated that immunosuppression has to occur concurrent with or very short after infection with *A. fumigatus* to facilitate clinical disease (Mircescu et al., 2009).

In context with dissemination of infection, there was presence of fungus observed in brain, yet, it is uncertain whether the dissemination necessarily leads to infection of organs as the fungus was not found viable. It is reported that hyphae break off during angioinvasion and so the vessels can move to other organs (Dagenais and Keller, 2009). Under what circumstances, however angioinvasion to hematogenous spread occurs and whether this occurs only temporarily or over the entire course of infection remains unclear and could not be answered with this mice model of *A. fumigatus* infection and needs to be analyzed further. Additionally, PCR being a very sensitive method results in detection of minimal number of fungal pathogens (Loeffler et al., 2001) unlike other methods. The non viability of fungus in brain could be due to the cytoxic effects arising from immune responses.

### 4.2.2 Dissemination of *A. fumigatus* infection in immune competent mice

For immunocompetent mice, it is described that the pulmonary infection usually is resolved within 24 h (Armstrong-James 2009) and inhaled conidia are cleared efficiently (Ibrahim-Granet et al. 2003) by recruitment of immune cells like neutrophils and macrophages due to which the fungal burden decreases over time (Duong et al. 1998; Brieland et al. 2001). However, few *A. fumigatus* conidia still persist in the lung (Mircescu et al. 2009). So far, however, it remains unclear whether this infection spreads to other organs.

In immunocompetent mice, it was shown that mice survive after intranasal infection with $10^7$ conidia up to 12 days post infection without any clinical symptoms (Armstrong James, 2009), so for this experimental group, a fungal dose of $10^6$ conidia was given to immunocompetent mice for the investigation of the dissemination on the basis of pulmonary invasive aspergillosis. The fungus was cleared within 24 hours from the lungs of infected mice as reported previously and no dissemination of infection was found in other organs as analyzed by quantitative PCR and colony forming units. In context with this study, it still remains unclear whether there is dissemination of fungus from pulmonary infection in immunocompetent mice and needs to be evaluated further. A higher fungal dose as well as other animal models could be considered for further investigations.

## 4.3 *In ovo* chick embryos and *in vitro* real-time molecular interactions as tools to characterize radiotracers for *Aspergillus fumigatus* infection

The role of nuclear medicine in diagnosis of fungal infections is currently limited due to unavailability of proven radiotracers for imaging of fungal infection. However, various radiotracers have been proposed for the diagnosis of fungal infections (Ruchel et al., 2001; Lupetti et al., 2005; Yang et al., 2009; Petrik et al., 2010). For screening of radiotracers, several *in vitro* and *in vivo* models have been used. However, detailed in vitro characterization of novel PET-tracers constitutes a cost and time saving method to qualify their use *in vivo* on animals and humans. Here, for the first time, *in vivo* PET/CT imaging of *in ovo* chick embryos infected with *A. fumigatus* and *in vitro* real-time monitoring of tracer uptake by fungus was demonstrated using Ligand Tracer® technology. Ligand tracer® system provides an easy and precise method for real-time data acquisition of tracer uptake and has not being used for infection studies. Hence, in this study, Ligand tracer technology was optimized for *in vitro* characterization of radiotracer by fungus grown on cells. Recently, embryonated chicken eggs were used as an alternative model for *A. fumigatus* infection (Jacobsen et al., 2010). Furthermore, micro PET/CT imaging was applied as imaging technique for metabolic studies e.g, of bone formation in embryogenesis at day 13 until day 18 via fluorine-18 (Heidrich et al., 2012). Hence, besides *in vitro* characterization of radiotracer by Ligand Tracer® system, embryonated chicken eggs were used as model for *in vivo* characterization of radiotracers during PET/CT imaging of *A. fumigatus* infection. The imaging data for the presence of radiotracer signal was correlated with the fungal burden present at the region of interest. PET/CT imaging of *A. fumigatus* infection was demonstrated on CAM of embryonated chicken eggs and analyzed for the presence of fungal burden on CAM. Both the methods combined together can prove useful to speed up the screening of novel radiotracers for diagnosis of *A. fumigatus* infection in host. As a proof of principle, gallium-68 labeled siderophore triacetylfusarinine C ($^{68}$Ga-TAFC), which was reported as a novel radiotracer in rat model, (Petrick et al., 2010) was used in the study.

### 4.3.1 *In vitro* characterization of radiotracer to follow *A. fumigatus* infection by monitoring the uptake of $^{68}$Ga-TAFC.

The Ligand Tracer® system is a simple and robust technical system, extensively used in real-time visualization of molecular interactions. Comparatively, the interaction of the radioactive labeled tracer in an active region (sown cell) and an in situ-reference region in a cell culture plate can be shown. The position of the detector above the cell culture ensures the reading of the interacting target molecule(*Technical Notes Ridgeview Instruments*). Extensive applications of this system have been demonstrated in various experiments. Comparative studies of different cell lines, the representation of protein-protein-interactions and detection of radiolabeled antibodies belong to the multiple application area as well as the implementation of cell-based survival studies (Bjorke & Andersson, 2006) and the representation of glucose metabolism by using the analogue [$^{18}$F]-FDG radiotracer (*Technical Notes Ridgeview Instruments*)

The ease of use, the minimum requirement for radioactive tracer to be used and the time-saving mode of operation make the system ideal for advanced applications. Based on the manufacturer´s data, *A. fumigatus in vitro* infection model was adapted to the Ligand Tracer®-system. Parameters such as temperature, the experimental procedure and use of different media could be modified as per manufacturer's instructions and were applied successfully.

To evaluate the radiotracer *in vitro* using Ligand Tracer technology, the fungus was first grown on various solid agar media but due to complexity of media, the results could not be evaluated. The fungus was grown on A549 cells and the different concentration of fungus was used. An increased infection dose of 10 fungal spores per cell showed detachment of cell layers, so infection dose was adjusted to 5 fungal spores per cell. However, in context with the infection doses, various time periods were also evaluated. But since *A. fumigatus* starts germinating after a time period of about 6 hours, the time period of 12 h post infection was selected to ensure the mycelial growth over the cells.

The interaction of fungus with cells is influenced by temperature. A549 cells as well as *A. fumigatus* are grown usually grown at +37 °C. Any deviation from this temperature is

associated with negative effects on the growth of the cultures. For this reason, the set temperature of the device was raised from +25°C to +37°C during the adaptation experiments. Particularly to the study of cellular interactions and absorption processes, the physiological measurement conditions are essential. Concomitant increase of evaporation rates of the approach could not be observed within relatively short data acquisition times. In long-term studies, however, this phenomenon can have decisive influence and should be studied experimentally in advance which can be remedied by the transfer of the measurement setup from the lamina flow into the incubator. In the context of biological infection-L2-investigations, the transfer is not done.

Within mentioned temperature-adaption experiments, the delayed temperature setting in the system was critically evaluated. Only after the start of data recording, the specified target temperature was established. The implementation of a 10 minute free run, the measured temperature was equilibrated at +37°C before the tracer was added and the data collection started. Thus, deviations from the normal temperature could be avoided. Furthermore, the free run can be considered as an additive of the washing step with 1x PBS buffer treatment of the cells. Furthermore, the use of different medium for tracer dilution need to be considered. Use of tracer with 1x PBS showed a decreased uptake of radiotracer which could be possibly due to insufficient availability of nutrients. However, when RPMI media was used without fetal calf serum (FCS), the results could be reproduced and hence set for Ligand Tracer® technology in context with *A. fumigatus* and A549 cells.

In the literature, open culture conditions through the use of Petri dishes are usually associated with higher risk of contaminations and possible physiological effects to the cells. To avoid those contaminations, the system was run under sterile conditions using sterile bench. Contrary to the manufacturer´s data, the volume of the culture medium was increased to 4 ml in the Petri dish. After standardisation of protocol for radiotracer uptake using Ligand Tracer technology, the system can be further evaluated to trace the uptake of radionucleootides using different mutants. For example, use of *hapX* mutant showed a decrease in uptake of $^{68}$Ga-TAFC compared with wild type strain of *A. fumigatus*.

A high priority is attached to the *in vitro* Ligand Tracer® system for the characterization of real-time data acquisition of tracer uptake. Fundamental interaction studies offer the

possibility to characterize tracers in a low-cost, time-saving and efficient manner. Compared to other assays, Ligand Tracer®system offers automated assaying on single cell preparations which reduces the errors and variations. Profitable insights can be obtained for the *in vitro* interaction of newly generated tracers and the transfer to the *in vivo* system can be postponed, such as for PET/CT examinations.

### 4.3.2 *In ovo* characterization of radiotracer using embryonated chicken eggs as infection model to follow *A. fumigatus* infection using $^{68}$Ga-TAFC

The detailed characterization and development of novel diagnostic radiotracers for tracing *Aspergillus* infections depends on the availability of suitable *in vivo* models. Additionally, to validate the *in vitro* screening test of newly designed radiotracers, various *in vivo* models like rodents play a major role. Though these models are classified as gold standard models, however, their usage is limited due to ethical issues. Further, these models require specialized skills and facilities and bear high cost. Due to these limitations, alternative models such as chicken egg model can be used to overcome these drawbacks.

The use of embryonated eggs infected with fungal pathogens via CAM helped to develop the infection on vascular layer of the egg within 2 days depending on the infection doses. A higher infection dose is able to invade the tissue and blood vessels which resemble histological findings for human patients and experimentally infected mice (Jacobsen et al., 2010). Macroscopically, it was found that the fungal growth on CAM was not only localized to the site of infection but there were few other fungal spots too found on CAM which were not described in other studies. However, the presence of fungal burden was described on CAM where the fungus was not visualized macroscopically (Jacoben et al., 2010). Also, infection doses upto $10^4$ showed no dissemination of infection to liver as was also described before (Jacobsen et al., 2010).

Imaging of *A. fumigatus* infection in rats using gallium labeled siderophores has been proposed as a useful tracer (Petrick et al., 2010). In this study, $^{68}$Ga-TAFC was used and injected via albumen into the egg embryos. To avoid the interference of the motion artefacts during imaging, eggs were anesthetized by using isofluorane (Heidrich et al., 2011). After injection of radiotracer, imaging was done and tracer uptake was quantified at regions of

interest. The tracer uptake when correlated with fungal burden were similar and showed higher tracer uptake as well as higher fungal burden at the site on infection.

Although there are no dynamic ways of measurements of tracer uptake in small animals, this method could be used in testing novel radiotracers for diagnosis of *Aspergillus* infection. The availability of chicken embryos as an *in vivo* model for screening radiotracers can prove beneficial when considering the initial screening of the radiotracers. Chicken chorioallantoic membrane model has been used as new tool in oncology imaging (Warnock et al., 2013). The ability to visualize infections rapidly makes it possible to verify new PET tracers for *Aspergillus* infection which finally can find way to animal studies. This can directly lead to the reduction of animal models as per the principles of 3Rs (reduction, replacement and refinement). However, it can't be ignored that embryonated chicken model cannot fully replace the rodent models for tracing *Aspergillus* infection but the embryonated egg model can provide a useful tool to screen novel radiotracers for diagnosis of *A. fumigatus* infection using PET/CT imaging before applying to murine models.

## 4.4 Conclusions

The work presented in this thesis provides insight into the host-pathogen interactions at molecular level involving epithelial cells as well as at organism level involving mice and chicken eggs. At molecular level, the first barrier to invading *A. fumigatus* conidia is the layer of epithelial cells. This part of my thesis indicates that *A. fumigatus* forms niche inside epithelial cells for its survival by inhibiting the host cell apoptosis and that conidial melanin is essential for the anti-apoptotic effect in alveolar epithelial cells. For the first time, the observations made here show that melanin plays an important role in uptake of conidia by epithelial cells and this effect was unlike macrophages which might be due to different receptors specific for melanin.

At organism level, infection models are essential in clinical and research sciences to evaluate various aspects of pathogenecity of microorganisms. Here, mice model was evaluated to follow the dissemination of infection after lung infection but there was no clear indication of fungal dissemination and further evaluation needs to be considered with other animal models or with different fungal doses and time points. Other infection model, chicken embryo, was used as cost effective and ethically favorable test system to evaluate novel radiotracers for imaging *A. fumigatus* infection via PET/CT analysis. The protocol was established and could be used further with other radiotracers. Since PET/CT is used as clinical and preclinal technique, the use of chick embryos can be helpful in screening novel radiotracers which can contribute to human medicine.

Ligan Tracer® is a novel technology used for in vitro assaying of radiotracers and here, for the first time, it was used as system for infectious assays with *A. fumigatus.* The protocol could be established and can further be used as test system with novel radiotracers and newly developed fungal mutants.

# References

**Aimanianda, V., Bayry, J., Bozza, S., Kniemeyer, O., Perruccio, K., Elluru, S. R., Clavaud, C., Paris, S., Brakhage, A. A., Kaveri, S. V., Romani, L., and Latge, J. P.,** 2009, "Surface hydrophobin prevents immune recognition of airborne fungal spores," Nature, 460(7259), pp. 1117-1121.

**Alekseeva, L., Huet, D., Femenia, F., Mouyna, I., Abdelouahab, M., Cagna, A., Guerrier, D., Tichanne-Seltzer, V., Baeza-Squiban, A., Chermette, R., Latge, J. P., and Berkova, N.,** 2009, "Inducible expression of beta defensins by human respiratory epithelial cells exposed to Aspergillus fumigatus organisms," BMC microbiology, 9, p. 33.

**Almyroudis, N. G., Holland, S. M., and Segal, B. H.,** 2005, "Invasive aspergillosis in primary immunodeficiencies," Medical mycology : official publication of the International Society for Human and Animal Mycology, 43 Suppl 1, pp. S247-259.

**Alp, S., and Arikan, S.,** 2008, "Investigation of extracellular elastase, acid proteinase and phospholipase activities as putative virulence factors in clinical isolates of Aspergillus species," Journal of basic microbiology, 48(5), pp. 331-337.

**Altschuh, D., Bjorkelund, H., Strandgard, J., Choulier, L., Malmqvist, M., and Andersson, K.,** 2012, "Deciphering complex protein interaction kinetics using Interaction Map," Biochemical and biophysical research communications, 428(1), pp. 74-79.

**Amitani, R., and Kawanami, R.,** 2009, "Interaction of Aspergillus with human respiratory mucosa: a study with organ culture model," Medical mycology : official publication of the International Society for Human and Animal Mycology, 47 Suppl 1, pp. S127-131.

**Armstrong-James, D. P., Turnbull, S. A., Teo, I., Stark, J., Rogers, N. J., Rogers, T. R., Bignell, E., and Haynes, K.,** 2009, "Impaired interferon-gamma responses, increased interleukin-17 expression, and a tumor necrosis factor-alpha transcriptional program in invasive aspergillosis," The Journal of infectious diseases, 200(8), pp. 1341-1351.

**Askew, D. S.,** 2008, "Aspergillus fumigatus: virulence genes in a street-smart mold," Curr Opin Microbiol, 11(4), pp. 331-337.

**Balloy, V., Sallenave, J. M., Wu, Y., Touqui, L., Latge, J. P., Si-Tahar, M., and Chignard, M.,** 2008, "Aspergillus fumigatus-induced interleukin-8 synthesis by respiratory epithelial cells is controlled by the phosphatidylinositol 3-kinase, p38 MAPK, and ERK1/2 pathways and not by the toll-like receptor-MyD88 pathway," The Journal of biological chemistry, 283(45), pp. 30513-30521.

**Banerjee, B., Greenberger, P. A., Fink, J. N., and Kurup, V. P.,** 1998, "Immunological characterization of Asp f 2, a major allergen from Aspergillus fumigatus associated with allergic bronchopulmonary aspergillosis," Infect Immun, 66(11), pp. 5175-5182.

**Banga, S., Gao, P., Shen, X., Fiscus, V., Zong, W. X., Chen, L., and Luo, Z. Q.,** 2007, "Legionella pneumophila inhibits macrophage apoptosis by targeting pro-death members of the Bcl2 protein family," Proceedings of the National Academy of Sciences of the United States of America, 104(12), pp. 5121-5126.

**Barta, P., Malmberg, J., Melicharova, L., Strandgard, J., Orlova, A., Tolmachev, V., Laznicek, M., and Andersson, K.,** 2012, "Protein interactions with HER-family receptors can have different characteristics depending on the hosting cell line," International journal of oncology, 40(5), pp. 1677-1682.

**Beffa, T., Staib, F., Lott Fischer, J., Lyon, P. F., Gumowski, P., Marfenina, O. E., Dunoyer-Geindre, S., Georgen, F., Roch-Susuki, R., Gallaz, L., and Latge, J. P.,** 1998, "Mycological control and surveillance of biological waste and compost," Medical mycology : official publication of the International Society for Human and Animal Mycology, 36 Suppl 1, pp. 137-145.

**Behnsen, J., Hartmann, A., Schmaler, J., Gehrke, A., Brakhage, A. A., and Zipfel, P. F.**, 2008, "The opportunistic human pathogenic fungus Aspergillus fumigatus evades the host complement system," Infect Immun, 76(2), pp. 820-827.

**Beisswenger, C., Hess, C., and Bals, R.,** 2012, "Aspergillus fumigatus conidia induce interferon-beta signalling in respiratory epithelial cells," Eur Respir J, 39(2), pp. 411-418.

**Bellamy, C. O., Malcomson, R. D., Harrison, D. J., and Wyllie, A. H.,** 1995, "Cell death in health and disease: the biology and regulation of apoptosis," Seminars in cancer biology, 6(1), pp. 3-16.

**Bellanger, A. P., Millon, L., Khoufache, K., Rivollet, D., Bieche, I., Laurendeau, I., Vidaud, M., Botterel, F., and Bretagne, S.,** 2009, "Aspergillus fumigatus germ tube growth and not conidia ingestion induces expression of inflammatory mediator genes in the human lung epithelial cell line A549," Journal of medical microbiology, 58(Pt 2), pp. 174-179.

**Ben-Ami, R., Lamaris, G. A., Lewis, R. E., and Kontoyiannis, D. P.,** 2010, "Interstrain variability in the virulence of Aspergillus fumigatus and Aspergillus terreus in a Toll-deficient Drosophila fly model of invasive aspergillosis," Medical mycology : official publication of the International Society for Human and Animal Mycology, 48(2), pp. 310-317.

**Berenguer, J., Allende, M. C., Lee, J. W., Garrett, K., Lyman, C., Ali, N. M., Bacher, J., Pizzo, P. A., and Walsh, T. J.,** 1995, "Pathogenesis of pulmonary aspergillosis. Granulocytopenia versus cyclosporine and methylprednisolone-induced immunosuppression," American journal of respiratory and critical care medicine, 152(3), pp. 1079-1086.

**Berkova, N., Lair-Fulleringer, S., Femenia, F., Huet, D., Wagner, M. C., Gorna, K., Tournier, F., Ibrahim-Granet, O., Guillot, J., Chermette, R., Boireau, P., and Latge, J. P.,** 2006, "Aspergillus fumigatus conidia inhibit tumour necrosis factor- or staurosporine-induced apoptosis in epithelial cells," Int Immunol, 18(1), pp. 139-150.

**Bermudez, L. E., and Goodman, J.,** 1996, "Mycobacterium tuberculosis invades and replicates within type II alveolar cells," Infect Immun, 64(4), pp. 1400-1406.

**Bernard, M., and Latge, J. P.,** 2001, "Aspergillus fumigatus cell wall: composition and biosynthesis," Medical mycology : official publication of the International Society for Human and Animal Mycology, 39 Suppl 1, pp. 9-17.

**Bhabhra, R., and Askew, D. S.,** 2005, "Thermotolerance and virulence of Aspergillus fumigatus: role of the fungal nucleolus," Medical mycology : official publication of the International Society for Human and Animal Mycology, 43 Suppl 1, pp. S87-93.

**Bhardwaj, A., and Aggarwal, B. B.,** 2003, "Receptor-mediated choreography of life and death," Journal of clinical immunology, 23(5), pp. 317-332.

**Bjorke, H., and Andersson, K.,** 2006, "Measuring the affinity of a radioligand with its receptor using a rotating cell dish with in situ reference area," Applied radiation and isotopes : including data, instrumentation and methods for use in agriculture, industry and medicine, 64(1), pp. 32-37.

**Bjorke, H., and Andersson, K.,** 2006, "Automated, high-resolution cellular retention and uptake studies in vitro," Applied radiation and isotopes : including data, instrumentation and methods for use in agriculture, industry and medicine, 64(8), pp. 901-905.

**Bjorkelund, H., Gedda, L., and Andersson, K.,** 2011, "Comparing the epidermal growth factor interaction with four different cell lines: intriguing effects imply strong dependency of cellular context," PloS one, 6(1), p. e16536.

**Bjorkelund, H., Gedda, L., and Andersson, K.,** 2011, "Avoiding false negative results in specificity analysis of protein-protein interactions," Journal of molecular recognition : JMR, 24(1), pp. 81-89.

**Bjorkelund, H., Gedda, L., Barta, P., Malmqvist, M., and Andersson, K.,** 2011, "Gefitinib induces epidermal growth factor receptor dimers which alters the interaction characteristics with (1)(2)(5)I-EGF," PloS one, 6(9).

**Bodey, G., Bueltmann, B., Duguid, W., Gibbs, D., Hanak, H., Hotchi, M., Mall, G., Martino, P., Meunier, F., Milliken, S., and et al.,** 1992, "Fungal infections in cancer patients: an international autopsy survey," European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology, 11(2), pp. 99-109.

**Boerman, O. C., Laverman, P., Oyen, W. J., Corstens, F. H., and Storm, G.,** 2000, "Radiolabeled liposomes for scintigraphic imaging," Progress in lipid research, 39(5), pp. 461-475.

**Bouchara, J. P., Sanchez, M., Chevailler, A., Marot-Leblond, A., Lissitzky, J. C., Tronchin, G., and Chabasse, D.,** 1997, "Sialic acid-dependent recognition of laminin and fibrinogen by Aspergillus fumigatus conidia," Infect Immun, 65(7), pp. 2717-2724.

**Bowman, J. C., Abruzzo, G. K., Anderson, J. W., Flattery, A. M., Gill, C. J., Pikounis, V. B., Schmatz, D. M., Liberator, P. A., and Douglas, C. M.,** 2001, "Quantitative PCR assay to measure Aspergillus fumigatus burden in a murine model of disseminated aspergillosis: demonstration of efficacy of caspofungin acetate," Antimicrob Agents Chemother, 45(12), pp. 3474-3481.

**Brakhage, A. A.,** 2005, "Systemic fungal infections caused by Aspergillus species: epidemiology, infection process and virulence determinants," Curr Drug Targets, 6(8), pp. 875-886.

**Brakhage, A. A., Bruns, S., Thywissen, A., Zipfel, P. F., and Behnsen, J.,** 2010, "Interaction of phagocytes with filamentous fungi," Curr Opin Microbiol, 13(4), pp. 409-415.

**Brakhage, A. A., and Langfelder, K.,** 2002, "Menacing mold: the molecular biology of Aspergillus fumigatus," Annu Rev Microbiol, 56, pp. 433-455.

**Brakhage, A. A., and Liebmann, B.,** 2005, "Aspergillus fumigatus conidial pigment and cAMP signal transduction: significance for virulence," Medical mycology : official publication of the International Society for Human and Animal Mycology, 43 Suppl 1, pp. S75-82.

**Bretagne, S.,** 2003, "Molecular diagnostics in clinical parasitology and mycology: limits of the current polymerase chain reaction (PCR) assays and interest of the real-time PCR assays," Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases, 9(6), pp. 505-511.

**Brieland, J. K., Jackson, C., Menzel, F., Loebenberg, D., Cacciapuoti, A., Halpern, J., Hurst, S., Muchamuel, T., Debets, R., Kastelein, R., Churakova, T., Abrams, J., Hare, R., and O'Garra, A.,** 2001, "Cytokine networking in lungs of immunocompetent mice in response to inhaled Aspergillus fumigatus," Infect Immun, 69(3), pp. 1554-1560.

**Brock, M., Jouvion, G., Droin-Bergere, S., Dussurget, O., Nicola, M. A., and Ibrahim-Granet, O.,** 2008, "Bioluminescent Aspergillus fumigatus, a new tool for drug efficiency testing and in vivo monitoring of invasive aspergillosis," Applied and environmental microbiology, 74(22), pp. 7023-7035.

**Bromley, I. M., and Donaldson, K.,** 1996, "Binding of Aspergillus fumigatus spores to lung epithelial cells and basement membrane proteins: relevance to the asthmatic lung," Thorax, 51(12), pp. 1203-1209.

**Brown, G. D., Taylor, P. R., Reid, D. M., Willment, J. A., Williams, D. L., Martinez-Pomares, L., Wong, S. Y., and Gordon, S.,** 2002, "Dectin-1 is a major beta-glucan receptor on macrophages," J Exp Med, 196(3), pp. 407-412.
[45] Carmody, R. J., and Cotter, T. G., 2001, "Signalling apoptosis: a radical approach," Redox Rep, 6(2), pp. 77-90.

**Bruns, S., Kniemeyer, O., Hasenberg, M., Aimanianda, V., Nietzsche, S., Thywissen, A., Jeron, A., Latge, J. P., Brakhage, A. A., and Gunzer, M.,** 2010, "Production of extracellular traps against Aspergillus fumigatus in vitro and in infected lung tissue is dependent on invading neutrophils and influenced by hydrophobin RodA," PLoS Pathog, 6(4), p. e1000873.

**Casadevall, A.,** 2005, "Fungal virulence, vertebrate endothermy, and dinosaur extinction: is there a connection?," Fungal Genet Biol, 42(2), pp. 98-106.

**Chai, L. Y., Vonk, A. G., Kullberg, B. J., Verweij, P. E., Verschueren, I., van der Meer, J. W., Joosten, L. A., Latge, J. P., and Netea, M. G.,** 2011, "Aspergillus fumigatus cell wall components differentially modulate host TLR2 and TLR4 responses," Microbes Infect, 13(2), pp. 151-159.

**Chandra, J., Samali, A., and Orrenius, S.,** 2000, "Triggering and modulation of apoptosis by oxidative stress," Free Radic Biol Med, 29(3-4), pp. 323-333.

**Chose, O., Sarde, C. O., Noel, C., Gerbod, D., Jimenez, J. C., Brenner, C., Capron, M., Viscogliosi, E., and Roseto, A.,** 2003, "Cell death in protists without mitochondria," Ann N Y Acad Sci, 1010, pp. 121-125.

**Clark, C. S., and Maurelli, A. T.,** 2007, "Shigella flexneri inhibits staurosporine-induced apoptosis in epithelial cells," Infect Immun, 75(5), pp. 2531-2539.

**Clemons, K. V., and Stevens, D. A.,** 2005, "The contribution of animal models of aspergillosis to understanding pathogenesis, therapy and virulence," Medical mycology : official publication of the International Society for Human and Animal Mycology, 43 Suppl 1, pp. S101-110.

**Commoner, B., Townsend, J., and Pake, G. E.,** 1954, "Free radicals in biological materials," Nature, 174(4432), pp. 689-691.

**Cordonnier, C., Gilain, L., Ricolfi, F., Deforges, L., Girard-Pipau, F., Poron, F., Millepied, M. C., and Escudier, E.,** 1996, "Acquired ciliary abnormalities of nasal mucosa in marrow recipients," Bone marrow transplantation, 17(4), pp. 611-616.

**Dagenais, T. R., and Keller, N. P.,** 2009, "Pathogenesis of Aspergillus fumigatus in Invasive Aspergillosis," Clin Microbiol Rev, 22(3), pp. 447-465.

**Dague, E., Alsteens, D., Latge, J. P., and Dufrene, Y. F.,** 2008, "High-resolution cell surface dynamics of germinating Aspergillus fumigatus conidia," Biophysical journal, 94(2), pp. 656-660.

**de Cassia, R. G. R., and Pombeiro-Sponchiado, S. R.,** 2005, "Antioxidant activity of the melanin pigment extracted from Aspergillus nidulans," Biological & pharmaceutical bulletin, 28(6), pp. 1129-1131.

**DeHart, D. J., Agwu, D. E., Julian, N. C., and Washburn, R. G.,** 1997, "Binding and germination of Aspergillus fumigatus conidia on cultured A549 pneumocytes," The Journal of infectious diseases, 175(1), pp. 146-150.

**Del Val Gomez, M., Gallardo, F. G., Babe, J., and Cobo, J.,** 2000, "Ga-67 scan in invasive aspergillosis: a case report," Clinical nuclear medicine, 25(12), pp. 1035-1036.

**D'Enfert, C., Diaquin, M., Delit, A., Wuscher, N., Debeaupuis, J. P., Huerre, M., and Latge, J. P.,** 1996, "Attenuated virulence of uridine-uracil auxotrophs of Aspergillus fumigatus," Infect Immun, 64(10), pp. 4401-4405.

**Denning, D. W.,** 1994, "Aspergillus tracheobronchitis," Clin Infect Dis, 19(6), pp. 1176-1177.

**Denning, D. W.,** 1998, "Invasive aspergillosis," Clin Infect Dis, 26(4), pp. 781-803; quiz 804-785.

**Denning, D. W., Marinus, A., Cohen, J., Spence, D., Herbrecht, R., Pagano, L., Kibbler, C., Kcrmery, V., Offner, F., Cordonnier, C., Jehn, U., Ellis, M., Collette, L., and Sylvester, R.,** 1998, "An EORTC multicentre prospective survey of invasive aspergillosis in haematological patients: diagnosis and therapeutic outcome. EORTC Invasive Fungal Infections Cooperative Group," The Journal of infection, 37(2), pp. 173-180.

**Dixon, D. M., McNeil, M. M., Cohen, M. L., Gellin, B. G., and La Montagne, J. R.,** 1996, "Fungal infections: a growing threat," Public health reports (Washington, D.C. : 1974), 111(3), pp. 226-235.

**Dixon, D. M., Polak, A., and Walsh, T. J.,** 1989, "Fungus dose-dependent primary pulmonary aspergillosis in immunosuppressed mice," Infect Immun, 57(5), pp. 1452-1456.

**Drakos, P. E., Nagler, A., Or, R., Naparstek, E., Kapelushnik, J., Engelhard, D., Rahav, G., Ne'emean, D., and Slavin, S.,** 1993, "Invasive fungal sinusitis in patients undergoing bone marrow transplantation," Bone marrow transplantation, 12(3), pp. 203-208.

**Duong, M., Ouellet, N., Simard, M., Bergeron, Y., Olivier, M., and Bergeron, M. G.,** 1998, "Kinetic study of host defense and inflammatory response to Aspergillus fumigatus in steroid-induced immunosuppressed mice," The Journal of infectious diseases, 178(5), pp. 1472-1482.

**Eissenberg, L. G., Goldman, W. E., and Schlesinger, P. H.,** 1993, "Histoplasma capsulatum modulates the acidification of phagolysosomes," J Exp Med, 177(6), pp. 1605-1611.

**Ekerljung, L., Lennartsson, J., and Gedda, L.,** 2012, "The HER2-binding affibody molecule (Z(HER2ratio342))(2) increases radiosensitivity in SKBR-3 cells," PloS one, 7(11), p. e49579.

**Ekerljung, L., Wallberg, H., Sohrabian, A., Andersson, K., Friedman, M., Frejd, F. Y., Stahl, S., and Gedda, L.,** 2012, "Generation and evaluation of bispecific affibody molecules for simultaneous targeting of EGFR and HER2," Bioconjugate chemistry, 23(9), pp. 1802-1811.

**Ellis, D.,** 2002, "Amphotericin B: spectrum and resistance," The Journal of antimicrobial chemotherapy, 49 Suppl 1, pp. 7-10.

**Ellis, M.,** 1999, "Therapy of Aspergillus fumigatus-related diseases," Contributions to microbiology, 2, pp. 105-129.

**Faherty, C. S., and Maurelli, A. T.,** 2008, "Staying alive: bacterial inhibition of apoptosis during infection," Trends in microbiology, 16(4), pp. 173-180.

**Feldmesser, M.,** 2005, "Prospects of vaccines for invasive aspergillosis," Medical mycology : official publication of the International Society for Human and Animal Mycology, 43(7), pp. 571-587.

**Femenia, F., Huet, D., Lair-Fulleringer, S., Wagner, M. C., Sarfati, J., Shingarova, L., Guillot, J., Boireau, P., Chermette, R., and Berkova, N.,** 2009, "Effects of conidia of various Aspergillus species on apoptosis of human pneumocytes and bronchial epithelial cells," Mycopathologia, 167(5), pp. 249-262.

**Filler, S. G., and Sheppard, D. C.,** 2006, "Fungal invasion of normally non-phagocytic host cells," PLoS Pathog, 2(12), p. e129.

**Finlay, B. B., and Falkow, S.,** 1988, "Comparison of the invasion strategies used by Salmonella cholerae-suis, Shigella flexneri and Yersinia enterocolitica to enter cultured animal cells: endosome acidification is not required for bacterial invasion or intracellular replication," Biochimie, 70(8), pp. 1089-1099.

**Flannagan, R. S., Cosio, G., and Grinstein, S.,** 2009, "Antimicrobial mechanisms of phagocytes and bacterial evasion strategies," Nat Rev Microbiol, 7(5), pp. 355-366.

**Ford, S., and Friedman, L.,** 1967, "Experimental study of the pathogenicity of aspergilli for mice," Journal of bacteriology, 94(4), pp. 928-933.

**Fransson, J., and Borrebaeck, C. A.,** 2006, "The nuclear DNA repair protein Ku70/80 is a tumor-associated antigen displaying rapid receptor mediated endocytosis," International journal of cancer. Journal international du cancer, 119(10), pp. 2492-2496.

**Fratti, R. A., Backer, J. M., Gruenberg, J., Corvera, S., and Deretic, V., 2001**, "Role of phosphatidylinositol 3-kinase and Rab5 effectors in phagosomal biogenesis and mycobacterial phagosome maturation arrest," The Journal of cell biology, 154(3), pp. 631-644.

**Friedman, M., Lindstrom, S., Ekerljung, L., Andersson-Svahn, H., Carlsson, J., Brismar, H., Gedda, L., Frejd, F. Y., and Stahl, S.,** 2009, "Engineering and characterization of a bispecific HER2 x EGFR-binding affibody molecule," Biotechnology and applied biochemistry, 54(2), pp. 121-131.

**Gastebois, A., Clavaud, C., Aimanianda, V., and Latge, J. P.,** 2009, "Aspergillus fumigatus: cell wall polysaccharides, their biosynthesis and organization," Future Microbiol, 4(5), pp. 583-595.

**Gebhardt, P., Opfermann, T., and Saluz, H. P.,** 2010, "Computer controlled 68Ga milking and concentration system," Applied radiation and isotopes : including data, instrumentation and methods for use in agriculture, industry and medicine, 68(6), pp. 1057-1059.

**Gedda, L., Bjorkelund, H., and Andersson, K.,** 2010, "Real-time immunohistochemistry analysis of embedded tissue," Applied radiation and isotopes : including data, instrumentation and methods for use in agriculture, industry and medicine, 68(12), pp. 2372-2376.

**Gersuk, G. M., Underhill, D. M., Zhu, L., and Marr, K. A.,** 2006, "Dectin-1 and TLRs permit macrophages to distinguish between different Aspergillus fumigatus cellular states," Journal of immunology (Baltimore, Md. : 1950), 176(6), pp. 3717-3724.

**Giard, D. J., Aaronson, S. A., Todaro, G. J., Arnstein, P., Kersey, J. H., Dosik, H., and Parks, W. P.,** 1973, "In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors," Journal of the National Cancer Institute, 51(5), pp. 1417-1423.

**Gil, M. L., Penalver, M. C., Lopez-Ribot, J. L., O'Connor, J. E., and Martinez, J. P.,** 1996, "Binding of extracellular matrix proteins to Aspergillus fumigatus conidia," Infect Immun, 64(12), pp. 5239-5247.

**Gomez, B. L., and Nosanchuk, J. D.,** 2003, "Melanin and fungi," Current opinion in infectious diseases, 16(2), pp. 91-96.

**Gong, J., Traganos, F., and Darzynkiewicz, Z.,** 1994, "A selective procedure for DNA extraction from apoptotic cells applicable for gel electrophoresis and flow cytometry," Anal Biochem, 218(2), pp. 314-319.

**Gravelat, F. N., Beauvais, A., Liu, H., Lee, M. J., Snarr, B. D., Chen, D., Xu, W., Kravtsov, I., Hoareau, C. M., Vanier, G., Urb, M., Campoli, P., Al Abdallah, Q., et al.,** 2013, "Aspergillus galactosaminogalactan mediates adherence to host constituents and conceals hyphal beta-glucan from the immune system," PLoS Pathog, 9(8), p. e1003575.

**Gravelat, F. N., Ejzykowicz, D. E., Chiang, L. Y., Chabot, J. C., Urb, M., Macdonald, K. D., al-Bader, N., Filler, S. G., and Sheppard, D. C.,** 2010, "Aspergillus fumigatus MedA governs adherence, host cell interactions and virulence," Cell Microbiol, 12(4), pp. 473-488.

**Graybill, J. R., Kaster, S. R., and Drutz, D. J.,** 1983, "Treatment of experimental murine aspergillosis with BAY n7133," The Journal of infectious diseases, 148(5), pp. 898-906.

**Groll, A. H., Shah, P. M., Mentzel, C., Schneider, M., Just-Nuebling, G., and Huebner, K.,** 1996, "Trends in the postmortem epidemiology of invasive fungal infections at a university hospital," The Journal of infection, 33(1), pp. 23-32.

**Grosse, C., Heinekamp, T., Kniemeyer, O., Gehrke, A., and Brakhage, A. A.,** 2008, "Protein kinase A regulates growth, sporulation, and pigment formation in Aspergillus fumigatus," Applied and environmental microbiology, 74(15), pp. 4923-4933.

**Gugnani, H. C.,** 2003, "Ecology and taxonomy of pathogenic aspergilli," Frontiers in bioscience : a journal and virtual library, 8, pp. s346-357.

**Haas, H.,** 2003, "Molecular genetics of fungal siderophore biosynthesis and uptake: the role of siderophores in iron uptake and storage," Appl Microbiol Biotechnol, 62(4), pp. 316-330.

**Haas, A.,** 2007, "The phagosome: compartment with a license to kill," Traffic (Copenhagen, Denmark), 8(4), pp. 311-330.

**Hacker, G., Kirschnek, S., and Fischer, S. F.,** 2006, "Apoptosis in infectious disease: how bacteria interfere with the apoptotic apparatus," Medical microbiology and immunology, 195(1), pp. 11-19.

**Hanson, L. H., Clemons, K. V., Denning, D. W., and Stevens, D. A.,** 1995, "Efficacy of oral saperconazole in systemic murine aspergillosis," Journal of medical and veterinary mycology : bi-monthly publication of the International Society for Human and Animal Mycology, 33(5), pp. 311-317.

**Harkin, S. T., Cohen, G. M., and Gescher, A.,** 1998, "Modulation of apoptosis in rat thymocytes by analogs of staurosporine: lack of direct association with inhibition of protein kinase C," Molecular pharmacology, 54(4), pp. 663-670.

**Hartl, A., Sauerbrei, A., Stelzner, A., and Wutzler, P.,** 2004, "Influenza infection of the embryonated hen's egg/an alternative model for in vivo evaluation of antiviral compounds," Arzneimittelforschung, 54(2), pp. 130-134.

**Hayden, R. T., Isotalo, P. A., Parrett, T., Wolk, D. M., Qian, X., Roberts, G. D., and Lloyd, R. V.,** 2003, "In situ hybridization for the differentiation of Aspergillus, Fusarium, and Pseudallescheria species in tissue section," Diagnostic molecular pathology : the American journal of surgical pathology, part B, 12(1), pp. 21-26.

**Heidrich, A., Schmidt, J., Zimmermann, J., and Saluz, H. P.,** 2013, "Automated Segmentation and Object Classification of CT Images: Application to In Vivo Molecular Imaging of Avian Embryos," International Journal of Biomedical Imaging, 2013, p. 10.

**Heidrich, A., Wurbach, L., Opfermann, T., Gebhardt, P., and Saluz, H. P.,** 2012, "Three and four dimensional 18F fluoride microPET imaging of avian embryos in ovo," Biomed Tech (Berl).

**Heidrich, A., Würbach, L., Opfermann, T., and Saluz, H.,** 2011, "Motion-Artifact-Free In Vivo Imaging Utilizing Narcotized Avian Embryos In Ovo," Molecular Imaging and Biology, 13(2), pp. 208-214.

**Heinekamp, T., Thywissen, A., Macheleidt, J., Keller, S., Valiante, V., and Brakhage, A. A.,** 2012, "Aspergillus fumigatus melanins: interference with the host endocytosis pathway and impact on virulence," Front Microbiol, 3, p. 440.

**Higuchi, M., Singh, S., Chan, H., and Aggarwal, B. B.,** 1995, "Protease inhibitors differentially regulate tumor necrosis factor-induced apoptosis, nuclear factor-kappa B activation, cytotoxicity, and differentiation," Blood, 86(6), pp. 2248-2256.

**Hohl, T. M., Van Epps, H. L., Rivera, A., Morgan, L. A., Chen, P. L., Feldmesser, M., and Pamer, E. G.,** 2005, "Aspergillus fumigatus triggers inflammatory responses by stage-specific beta-glucan display," PLoS Pathog, 1(3), p. e30.

**Honarvar, H., Jokilaakso, N., Andersson, K., Malmberg, J., Rosik, D., Orlova, A., Karlstrom, A. E., Tolmachev, V., and Jarver, P.,** 2013, "Evaluation of backbone-cyclized HER2-binding 2-helix affibody molecule for in vivo molecular imaging," Nuclear medicine and biology, 40(3), pp. 378-386.

**Hope, W. W.,** 2009, "Invasion of the alveolar-capillary barrier by Aspergillus spp.: therapeutic and diagnostic implications for immunocompromised patients with invasive pulmonary aspergillosis," Medical mycology : official publication of the International Society for Human and Animal Mycology, 47 Suppl 1, pp. S291-298.

**Hope, W. W., Kruhlak, M. J., Lyman, C. A., Petraitiene, R., Petraitis, V., Francesconi, A., Kasai, M., Mickiene, D., Sein, T., et al.,** 2007, "Pathogenesis of Aspergillus fumigatus and the kinetics of galactomannan in an in vitro model of early invasive pulmonary aspergillosis: implications for antifungal therapy," The Journal of infectious diseases, 195(3), pp. 455-466.

**Hope, W. W., Walsh, T. J., and Denning, D. W.,** 2005, "Laboratory diagnosis of invasive aspergillosis," Lancet Infect Dis, 5(10), pp. 609-622.

**Hope, W. W., Walsh, T. J., and Denning, D. W.,** 2005, "The invasive and saprophytic syndromes due to Aspergillus spp," Medical mycology : official publication of the International Society for Human and Animal Mycology, 43 Suppl 1, pp. S207-238.

**Hot, A., Maunoury, C., Poiree, S., Lanternier, F., Viard, J. P., Loulergue, P., Coignard, H., Bougnoux, M. E., Suarez, F., Rubio, M. T., Mahlaoui, N., Dupont, B., Lecuit, M., Faraggi, M., and Lortholary, O.,** 2011, "Diagnostic contribution of positron emission tomography with [18F]fluorodeoxyglucose for invasive fungal infections," Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases, 17(3), pp. 409-417.

**Huffnagle, G. B., Chen, G. H., Curtis, J. L., McDonald, R. A., Strieter, R. M., and Toews, G. B.,** 1995, "Down-regulation of the afferent phase of T cell-mediated pulmonary inflammation and immunity by a high melanin-producing strain of Cryptococcus neoformans," Journal of immunology (Baltimore, Md. : 1950), 155(7), pp. 3507-3516.

**Ibata-Ombetta, S., Idziorek, T., Trinel, P. A., Poulain, D., and Jouault, T.,** 2003, "Candida albicans phospholipomannan promotes survival of phagocytosed yeasts through modulation of bad phosphorylation and macrophage apoptosis," The Journal of biological chemistry, 278(15), pp. 13086-13093.

**Ibrahim-Granet, O., Jouvion, G., Hohl, T. M., Droin-Bergere, S., Philippart, F., Kim, O. Y., Adib-Conquy, M., Schwendener, R., Cavaillon, J. M., and Brock, M.,** 2010, "In vivo bioluminescence imaging and histopathopathologic analysis reveal distinct roles for resident and recruited immune effector cells in defense against invasive aspergillosis," BMC microbiology, 10, p. 105.

**Jacobsen, I. D., Große, K., Berndt, A., and Hube, B.,** 2011, "Pathogenesis of Candida albicans Infections in the Alternative Chorio-Allantoic Membrane Chicken Embryo Model Resembles Systemic Murine Infections," PloS one, 6(5), p. e19741.

**Jacobsen, I. D., Grosse, K., and Hube, B.,** 2012, "Embryonated chicken eggs as alternative infection model for pathogenic fungi," Methods Mol Biol, 845, pp. 487-496.

**Jacobsen, I. D., Grosse, K., Slesiona, S., Hube, B., Berndt, A., and Brock, M.,** 2010, "Embryonated eggs as an alternative infection model to investigate Aspergillus fumigatus virulence," Infect Immun, 78(7), pp. 2995-3006.

**Jacobson, E. S.,** 2000, "Pathogenic roles for fungal melanins," Clin Microbiol Rev, 13(4), pp. 708-717.

**Jacobson, M. D.,** 1996, "Reactive oxygen species and programmed cell death," Trends Biochem Sci, 21(3), pp. 83-86.

**Jacobson, M. D., Weil, M., and Raff, M. C.,** 1997, "Programmed cell death in animal development," Cell, 88(3), pp. 347-354.

**Jahn, B., Boukhallouk, F., Lotz, J., Langfelder, K., Wanner, G., and Brakhage, A. A**., 2000, "Interaction of human phagocytes with pigmentless Aspergillus conidia," Infect Immun, 68(6), pp. 3736-3739.

**Jahn, B., Koch, A., Schmidt, A., Wanner, G., Gehringer, H., Bhakdi, S., and Brakhage, A. A.,** 1997, "Isolation and characterization of a pigmentless-conidium mutant of Aspergillus fumigatus with altered conidial surface and reduced virulence," Infect Immun, 65(12), pp. 5110-5117.

**Jahn, B., Langfelder, K., Schneider, U., Schindel, C., and Brakhage, A. A.,** 2002, "PKSP-dependent reduction of phagolysosome fusion and intracellular kill of Aspergillus fumigatus conidia by human monocyte-derived macrophages," Cell Microbiol, 4(12), pp. 793-803.

**Jia, X., Chen, F., Pan, W., Yu, R., Tian, S., Han, G., Fang, H., Wang, S., Zhao, J., Li, X., Zheng, D., Tao, S., Liao, W., Han, X., and Han, L.,** 2014, "Gliotoxin promotes Aspergillus fumigatus internalization into type II human pneumocyte A549 cells by inducing host phospholipase D activation," Microbes and infection / Institut Pasteur.

**Kawel, N., Schorer, G. M., Desbiolles, L., Seifert, B., Marincek, B., and Boehm, T.,** 2011, "Discrimination between invasive pulmonary aspergillosis and pulmonary lymphoma using CT," European journal of radiology, 77(3), pp. 417-425.

**Kemper, C. A., Hostetler, J. S., Follansbee, S. E., Ruane, P., Covington, D., Leong, S. S., Deresinski, S. C., and Stevens, D. A.,** 1993, "Ulcerative and plaque-like tracheobronchitis due to infection with Aspergillus in patients with AIDS," Clin Infect Dis, 17(3), pp. 344-352.

**Kim, M., Ashida, H., Ogawa, M., Yoshikawa, Y., Mimuro, H., and Sasakawa, C.,** 2010, "Bacterial interactions with the host epithelium," Cell host & microbe, 8(1), pp. 20-35.

**Klont, R. R., Mennink-Kersten, M. A., and Verweij, P. E.,** 2004, "Utility of Aspergillus antigen detection in specimens other than serum specimens," Clin Infect Dis, 39(10), pp. 1467-1474.

**Knodler, L. A., Finlay, B. B., and Steele-Mortimer, O.,** 2005, "The Salmonella effector protein SopB protects epithelial cells from apoptosis by sustained activation of Akt," The Journal of biological chemistry, 280(10), pp. 9058-9064.

**Krammer, P. H., Arnold, R., and Lavrik, I. N.,** 2007, "Life and death in peripheral T cells," Nature reviews. Immunology, 7(7), pp. 532-542.

**Krammer, P. H., Kaminski, M., Kiessling, M., and Gulow, K.,** 2007, "No life without death," Advances in cancer research, 97, pp. 111-138.

**Laemmli, U. K.,** 1970, "Cleavage of structural proteins during the assembly of the head of bacteriophage T4," Nature, 227(5259), pp. 680-685.

**Langfelder, K., Jahn, B., Gehringer, H., Schmidt, A., Wanner, G., and Brakhage, A. A**., 1998, "Identification of a polyketide synthase gene (pksP) of Aspergillus fumigatus involved in conidial pigment biosynthesis and virulence," Medical microbiology and immunology, 187(2), pp. 79-89.

**Langfelder, K., Streibel, M., Jahn, B., Haase, G., and Brakhage, A. A.,** 2003, "Biosynthesis of fungal melanins and their importance for human pathogenic fungi," Fungal Genet Biol, 38(2), pp. 143-158.

**Latge, J. P.,** 1999, "Aspergillus fumigatus and aspergillosis," Clin Microbiol Rev, 12(2), pp. 310-350.

**Latge, J. P.,** 2001, "The pathobiology of Aspergillus fumigatus," Trends in microbiology, 9(8), pp. 382-389.

**Latge, J. P.,** 2007, "The cell wall: a carbohydrate armour for the fungal cell," Mol Microbiol, 66(2), pp. 279-290.

**Latge, J. P.,** 2010, "Tasting the fungal cell wall," Cell Microbiol, 12(7), pp. 863-872.

**Latge, J. P., Mouyna, I., Tekaia, F., Beauvais, A., Debeaupuis, J. P., and Nierman, W.,** 2005, "Specific molecular features in the organization and biosynthesis of the cell wall of Aspergillus fumigatus," Medical mycology : official publication of the International Society for Human and Animal Mycology, 43 Suppl 1, pp. S15-22.

**Lewis, R. E., and Kontoyiannis, D. P.,** 2009, "Invasive aspergillosis in glucocorticoid-treated patients," Medical mycology : official publication of the International Society for Human and Animal Mycology, 47 Suppl 1, pp. S271-281.

**Lin, S. J., Schranz, J., and Teutsch, S. M.,** 2001, "Aspergillosis case-fatality rate: systematic review of the literature," Clin Infect Dis, 32(3), pp. 358-366.

**Lin, X., Kim, Y. A., Lee, B. L., Soderhall, K., and Soderhall, I.,** 2009, "Identification and properties of a receptor for the invertebrate cytokine astakine, involved in hematopoiesis," Experimental cell research, 315(7), pp. 1171-1180.

**Liu, G. Y., and Nizet, V.,** 2009, "Color me bad: microbial pigments as virulence factors," Trends in microbiology, 17(9), pp. 406-413.

**Loeffler, J., Hebart, H., Cox, P., Flues, N., Schumacher, U., and Einsele, H.,** 2001, "Nucleic acid sequence-based amplification of Aspergillus RNA in blood samples," Journal of clinical microbiology, 39(4), pp. 1626-1629.

**Lupetti, A., de Boer, M. G., Erba, P., Campa, M., and Nibbering, P. H.,** 2011, "Radiotracers for fungal infection imaging," Medical mycology : official publication of the International Society for Human and Animal Mycology, 49 Suppl 1, pp. S62-69.

**Lupetti, A., Welling, M. M., Mazzi, U., Nibbering, P. H., and Pauwels, E. K**., 2002, "Technetium-99m labelled fluconazole and antimicrobial peptides for imaging of Candida albicans and Aspergillus fumigatus infections," Eur J Nucl Med Mol Imaging, 29(5), pp. 674-679.

**Lupetti, A., Welling, M. M., Pauwels, E. K., and Nibbering, P. H.,** 2005, "Detection of fungal infections using radiolabeled antifungal agents," Curr Drug Targets, 6(8), pp. 945-954.

**Luther, K., Torosantucci, A., Brakhage, A. A., Heesemann, J., and Ebel, F.,** 2007, "Phagocytosis of Aspergillus fumigatus conidia by murine macrophages involves recognition by the dectin-1 beta-glucan receptor and Toll-like receptor 2," Cell Microbiol, 9(2), pp. 368-381.

**Malviya, G., de Vries, E. F., Dierckx, R. A., and Signore, A.,** 2011, "Synthesis and evaluation of 99mTc-labelled monoclonal antibody 1D09C3 for molecular imaging of major histocompatibility complex class II protein expression," Molecular imaging and biology : MIB : the official publication of the Academy of Molecular Imaging, 13(5), pp. 930-939.

**Marr, K. A., Patterson, T., and Denning, D.,** 2002, "Aspergillosis. Pathogenesis, clinical manifestations, and therapy," Infectious disease clinics of North America, 16(4), pp. 875-894, vi.

**Martinou, J. C., and Green, D. R.,** 2001, "Breaking the mitochondrial barrier," Nat Rev Mol Cell Biol, 2(1), pp. 63-67.

**Mbawuike, I. N., and Herscowitz, H. B.,** 1989, "MH-S, a murine alveolar macrophage cell line: morphological, cytochemical, and functional characteristics," Journal of leukocyte biology, 46(2), pp. 119-127.

**McDonagh, A., Fedorova, N. D., Crabtree, J., Yu, Y., Kim, S., Chen, D., Loss, O., Cairns, T., Goldman, G., Armstrong-James, D., Haynes, K., Haas, H., Schrettl, M., May, G., Nierman, W. C., and Bignell, E.,** 2008, "Sub-telomere directed gene expression during initiation of invasive aspergillosis," PLoS Pathog, 4(9), p. e1000154.

**Mech, F., Thywissen, A., Guthke, R., Brakhage, A. A., and Figge, M. T**., 2011, "Automated image analysis of the host-pathogen interaction between phagocytes and Aspergillus fumigatus," PloS one, 6(5), p. e19591.

**Mednick, A. J., Nosanchuk, J. D., and Casadevall, A**., 2005, "Melanization of Cryptococcus neoformans affects lung inflammatory responses during cryptococcal infection," Infect Immun, 73(4), pp. 2012-2019.

**Meier, P., Finch, A., and Evan, G.,** 2000, "Apoptosis in development," Nature, 407(6805), pp. 796-801.

**Mennink-Kersten, M. A., Donnelly, J. P., and Verweij, P. E.,** 2004, "Detection of circulating galactomannan for the diagnosis and management of invasive aspergillosis," Lancet Infect Dis, 4(6), pp. 349-357.

**Mennink-Kersten, M. A., Klont, R. R., Warris, A., Op den Camp, H. J., and Verweij, P. E.,** 2004, "Bifidobacterium lipoteichoic acid and false ELISA reactivity in aspergillus antigen detection," Lancet, 363(9405), pp. 325-327.

**Mennink-Kersten, M. A., Ruegebrink, D., Klont, R. R., Warris, A., Gavini, F., Op den Camp, H. J., and Verweij, P. E.,** 2005, "Bifidobacterial lipoglycan as a new cause for false-positive platelia Aspergillus enzyme-linked immunosorbent assay reactivity," Journal of clinical microbiology, 43(8), pp. 3925-3931.

**Mertens, K., Mees, G., Lambert, B., Van de Wiele, C., and Goethals, I.,** 2012, "In vitro 2-deoxy-2-[18F]fluoro-D-glucose uptake: practical considerations," Cancer biotherapy & radiopharmaceuticals, 27(3), pp. 183-188.

**Metcalf, S. C., and Dockrell, D. H.,** 2007, "Improved outcomes associated with advances in therapy for invasive fungal infections in immunocompromised hosts," The Journal of infection, 55(4), pp. 287-299.

**Mikulska, M., Raiola, A. M., Bruno, B., Furfaro, E., Van Lint, M. T., Bregante, S., Ibatici, A., Del Bono, V., Bacigalupo, A., and Viscoli, C.,** 2009, "Risk factors for invasive aspergillosis and related mortality in recipients of allogeneic SCT from alternative donors: an analysis of 306 patients," Bone marrow transplantation, 44(6), pp. 361-370.

**Miller, V. L., and Falkow, S.,** 1988, "Evidence for two genetic loci in Yersinia enterocolitica that can promote invasion of epithelial cells," Infect Immun, 56(5), pp. 1242-1248.

**Mircescu, M. M., Lipuma, L., van Rooijen, N., Pamer, E. G., and Hohl, T. M.,** 2009, "Essential role for neutrophils but not alveolar macrophages at early time points following Aspergillus fumigatus infection," The Journal of infectious diseases, 200(4), pp. 647-656.

**Morgan, J., Wannemuehler, K. A., Marr, K. A., Hadley, S., Kontoyiannis, D. P., Walsh, T. J., Fridkin, S. K., Pappas, P. G., and Warnock, D. W.,** 2005, "Incidence of invasive aspergillosis following hematopoietic stem cell and solid organ transplantation: interim results of a prospective multicenter surveillance program," Medical mycology : official publication of the International Society for Human and Animal Mycology, 43 Suppl 1, pp. S49-58.

**Morgan, M. A., Wilson, W. R., Neel, H. B., 3rd, and Roberts, G. D.,** 1984, "Fungal sinusitis in healthy and immunocompromised individuals," American journal of clinical pathology, 82(5), pp. 597-601.

**Mukaida, N.,** 2000, "Interleukin-8: an expanding universe beyond neutrophil chemotaxis and activation," International journal of hematology, 72(4), pp. 391-398.

**Nash, G., Irvine, R., Kerschmann, R. L., and Herndier, B.,** 1997, "Pulmonary aspergillosis in acquired immune deficiency syndrome: autopsy study of an emerging pulmonary complication of human immunodeficiency virus infection," Human pathology, 28(11), pp. 1268-1275.

**Nawada, R., Amitani, R., Tanaka, E., Niimi, A., Suzuki, K., Murayama, T., and Kuze, F.,** 1996, "Murine model of invasive pulmonary aspergillosis following an earlier stage, noninvasive Aspergillus infection," Journal of clinical microbiology, 34(6), pp. 1433-1439.

**Neofytos, D., Horn, D., Anaissie, E., Steinbach, W., Olyaei, A., Fishman, J., Pfaller, M., Chang, C., Webster, K., and Marr, K.,** 2009, "Epidemiology and outcome of invasive fungal infection in adult hematopoietic stem cell transplant recipients: analysis of Multicenter Prospective Antifungal Therapy (PATH) Alliance registry," Clin Infect Dis, 48(3), pp. 265-273.

**Nestor, M., Andersson, K., and Lundqvist, H.,** 2008, "Characterization of 111In and 177Lu-labeled antibodies binding to CD44v6 using a novel automated radioimmunoassay," Journal of molecular recognition : JMR, 21(3), pp. 179-183.

**Newman, S. L., Gootee, L., Hilty, J., and Morris, R. E.,** 2006, "Human macrophages do not require phagosome acidification to mediate fungistatic/fungicidal activity against Histoplasma capsulatum," Journal of immunology (Baltimore, Md. : 1950), 176(3), pp. 1806-1813.

**Novy, Z., Barta, P., Mandikova, J., Laznicek, M., and Trejtnar, F.,** 2012, "A comparison of in vitro methods for determining the membrane receptor expression in cell lines," Nuclear medicine and biology, 39(7), pp. 893-896.

**Osherov, N.,** 2012, "Interaction of the pathogenic mold Aspergillus fumigatus with lung epithelial cells," Front Microbiol, 3, p. 346.

**Pagano, L., Girmenia, C., Mele, L., Ricci, P., Tosti, M. E., Nosari, A., Buelli, M., Picardi, M., Allione, B., Corvatta, L., D'Antonio, D., et al.,** 2001, "Infections caused by filamentous fungi in patients with hematologic malignancies. A report of 391 cases by GIMEMA Infection Program," Haematologica, 86(8), pp. 862-870.

**Pagano, L., Ricci, P., Montillo, M., Cenacchi, A., Nosari, A., Tonso, A., Cudillo, L., Chierichini, A., Savignano, C., Buelli, M., Melillo, L., La Barbera, E. O., Sica, S., Hohaus, S., Bonini, A., Bucaneve, G., and Del Favero, A.,** 1996, "Localization of aspergillosis to the central nervous system among patients with acute leukemia: report of 14 cases. Gruppo Italiano Malattie Ematologiche dell'Adulto Infection Program," Clin Infect Dis, 23(3), pp. 628-630.

**Palmer, L. B., Greenberg, H. E., and Schiff, M. J**., 1991, "Corticosteroid treatment as a risk factor for invasive aspergillosis in patients with lung disease," Thorax, 46(1), pp. 15-20.

**Pardo, J., Urban, C., Galvez, E. M., Ekert, P. G., Muller, U., Kwon-Chung, J., Lobigs, M., Mullbacher, A., Wallich, R., Borner, C., and Simon, M. M.,** 2006, "The mitochondrial protein Bak is pivotal for gliotoxin-induced apoptosis and a critical host factor of Aspergillus fumigatus virulence in mice," The Journal of cell biology, 174(4), pp. 509-519.

**Paris, S., Boisvieux-Ulrich, E., Crestani, B., Houcine, O., Taramelli, D., Lombardi, L., and Latge, J. P**., 1997, "Internalization of Aspergillus fumigatus conidia by epithelial and endothelial cells," Infect Immun, 65(4), pp. 1510-1514.

**Paris, S., Wysong, D., Debeaupuis, J. P., Shibuya, K., Philippe, B., Diamond, R. D., and Latge, J. P.,** 2003, "Catalases of Aspergillus fumigatus," Infect Immun, 71(6), pp. 3551-3562.
[173] Park, S. J., and Mehrad, B., 2009, "Innate immunity to Aspergillus species," Clin Microbiol Rev, 22(4), pp. 535-551.

**Patterson, T. F.,** 2005, "The future of animal models of invasive aspergillosis," Medical mycology : official publication of the International Society for Human and Animal Mycology, 43 Suppl 1, pp. S115-119.

**Patterson, T. F., Kirkpatrick, W. R., White, M., Hiemenz, J. W., Wingard, J. R., Dupont, B., Rinaldi, M. G., Stevens, D. A., and Graybill, J. R.,** 2000, "Invasive aspergillosis. Disease spectrum, treatment practices, and outcomes. I3 Aspergillus Study Group," Medicine, 79(4), pp. 250-260.

**Pease, J. E., and Sabroe, I.,** 2002, "The role of interleukin-8 and its receptors in inflammatory lung disease: implications for therapy," American journal of respiratory medicine : drugs, devices, and other interventions, 1(1), pp. 19-25.

**Penalver, M. C., Casanova, M., Martinez, J. P., and Gil, M. L.,** 1996, "Cell wall protein and glycoprotein constituents of Aspergillus fumigatus that bind to polystyrene may be responsible for the cell surface hydrophobicity of the mycelium," Microbiology (Reading, England), 142 ( Pt 7), pp. 1597-1604.

**Penalver, M. C., O'Connor, J. E., Martinez, J. P., and Gil, M. L.,** 1996, "Binding of human fibronectin to Aspergillus fumigatus conidia," Infect Immun, 64(4), pp. 1146-1153.

**Petrik, M., Franssen, G. M., Haas, H., Laverman, P., Hortnagl, C., Schrettl, M., Helbok, A., Lass-Florl, C., and Decristoforo, C.,** 2012, "Preclinical evaluation of two 68Ga-siderophores as potential radiopharmaceuticals for Aspergillus fumigatus infection imaging," Eur J Nucl Med Mol Imaging, 39(7), pp. 1175-1183.

**Petrik, M., Haas, H., Dobrozemsky, G., Lass-Florl, C., Helbok, A., Blatzer, M., Dietrich, H., and Decristoforo, C**., 2010, "68Ga-siderophores for PET imaging of invasive pulmonary aspergillosis: proof of principle," Journal of nuclear medicine : official publication, Society of Nuclear Medicine, 51(4), pp. 639-645.

**Petrik, M., Haas, H., Schrettl, M., Helbok, A., Blatzer, M., and Decristoforo, C.,** 2012, "In vitro and in vivo evaluation of selected 68Ga-siderophores for infection imaging," Nuclear medicine and biology, 39(3), pp. 361-369.

**Pihet, M., Vandeputte, P., Tronchin, G., Renier, G., Saulnier, P., Georgeault, S., Mallet, R., Chabasse, D., Symoens, F., and Bouchara, J. P.,** 2009, "Melanin is an essential component for the integrity of the cell wall of Aspergillus fumigatus conidia," BMC microbiology, 9, p. 177.

**Post, M. J., Lass-Floerl, C., Gastl, G., and Nachbaur, D.,** 2007, "Invasive fungal infections in allogeneic and autologous stem cell transplant recipients: a single-center study of 166 transplanted patients," Transplant infectious disease : an official journal of the Transplantation Society, 9(3), pp. 189-195.

**Ramirez-Ortiz, Z. G., Specht, C. A., Wang, J. P., Lee, C. K., Bartholomeu, D. C., Gazzinelli, R. T., and Levitz, S. M.,** 2008, "Toll-like receptor 9-dependent immune activation by unmethylated CpG motifs in Aspergillus fumigatus DNA," Infect Immun, 76(5), pp. 2123-2129.

**Rath, P. C., and Aggarwal, B. B.,** 1999, "TNF-induced signaling in apoptosis," Journal of clinical immunology, 19(6), pp. 350-364.

**Reeves, E. P., Messina, C. G., Doyle, S., and Kavanagh, K.,** 2004, "Correlation between gliotoxin production and virulence of Aspergillus fumigatus in Galleria mellonella," Mycopathologia, 158(1), pp. 73-79.

**Rennen, H. J., Bleeker-Rovers, C. P., van Eerd, J. E., Frielink, C., Oyen, W. J., Corstens, F. H., and Boerman, O. C.,** 2004, "99mTc-labeled interleukin-8 for scintigraphic detection of pulmonary infections," Chest, 126(6), pp. 1954-1961.

**Reusser, P., Cordonnier, C., Einsele, H., Engelhard, D., Link, D., Locasciulli, A., and Ljungman, P.,** 1996, "European survey of herpesvirus resistance to antiviral drugs in bone marrow transplant recipients. Infectious Diseases Working Party of the European Group for Blood and Marrow Transplantation (EBMT)," Bone marrow transplantation, 17(5), pp. 813-817.

**Rhodes, J. C.,** 2006, "Aspergillus fumigatus: growth and virulence," Medical mycology : official publication of the International Society for Human and Animal Mycology, 44 Suppl 1, pp. S77-81.

**Ribaud, P., Chastang, C., Latge, J. P., Baffroy-Lafitte, L., Parquet, N., Devergie, A., Esperou, H., Selimi, F., Rocha, V., Esperou, H., Selimi, F., Rocha, V., Derouin, F., Socie, G., and Gluckman, E.,** 1999, "Survival and prognostic factors of invasive aspergillosis after allogeneic bone marrow transplantation," Clin Infect Dis, 28(2), pp. 322-330.

**Riley, P. A.,** 1997, "Melanin," The international journal of biochemistry & cell biology, 29(11), pp. 1235-1239.

**Rivera, A., Van Epps, H. L., Hohl, T. M., Rizzuto, G., and Pamer, E. G.,** 2005, "Distinct CD4+-T-cell responses to live and heat-inactivated Aspergillus fumigatus conidia," Infect Immun, 73(11), pp. 7170-7179.

**Rodrigues, V., Cordeiro-da-Silva, A., Laforge, M., Ouaissi, A., Silvestre, R., and Estaquier, J., 2012**, "Modulation of mammalian apoptotic pathways by intracellular protozoan parasites," Cell Microbiol, 14(3), pp. 325-333.

**Rouzet, F., Bachelet-Violette, L., Alsac, J. M., Suzuki, M., Meulemans, A., Louedec, L., Petiet, A., Jandrot-Perrus, M., Chaubet, F., Michel, J. B., Le Guludec, D., and Letourneur, D.,** 2011, "Radiolabeled fucoidan as a p-selectin targeting agent for in vivo imaging of platelet-rich thrombus and endothelial activation," Journal of nuclear medicine : official publication, Society of Nuclear Medicine, 52(9), pp. 1433-1440.

**Ruchel, R., Behe, M., Torp, B., and Laatsch, H.,** 2001, "Usefulness of optical brightners in medical mycology," Rev Iberoam Micol, 18(4), pp. 147-149.

**Rudel, T., Kepp, O., and Kozjak-Pavlovic, V.,** 2010, "Interactions between bacterial pathogens and mitochondrial cell death pathways," Nat Rev Microbiol, 8(10), pp. 693-705.

**Ruhland, A., Leal, N., and Kima, P. E**., 2007, "Leishmania promastigotes activate PI3K/Akt signalling to confer host cell resistance to apoptosis," Cell Microbiol, 9(1), pp. 84-96.

**Sarfati, J., Diaquin, M., Debeaupuis, J. P., Schmidt, A., Lecaque, D., Beauvais, A., and Latge, J. P**., 2002, "A new experimental murine aspergillosis model to identify strains of Aspergillus fumigatus with reduced virulence," Nihon Ishinkin Gakkai zasshi = Japanese journal of medical mycology, 43(4), pp. 203-213.

**Savill, J.,** 2000, "Apoptosis in resolution of inflammation," Kidney & blood pressure research, 23(3-5), pp. 173-174.

**Savill, J., and Fadok, V.,** 2000, "Corpse clearance defines the meaning of cell death," Nature, 407(6805), pp. 784-788.

**Schaffner, A.,** 1989, "Experimental basis for the clinical epidemiology of fungal infections. A review," Mycoses, 32(10), pp. 499-515.

**Schaffner, A.,** 1994, "Macrophage-Aspergillus interactions," Immunology series, 60, pp. 545-552.

**Schaffner, A., Douglas, H., and Braude, A.,** 1982, "Selective protection against conidia by mononuclear and against mycelia by polymorphonuclear phagocytes in resistance to Aspergillus. Observations on these two lines of defense in vivo and in vitro with human and mouse phagocytes," The Journal of clinical investigation, 69(3), pp. 617-631.

**Schaffner, A., Douglas, H., and Braude, A**., 1982, "Selective protection against conidia by mononuclear and against mycelia by polymorphonuclear phagocytes in resistance to Aspergillus. Observations on these two lines of defense in vivo and in vitro with human and mouse phagocytes," The Journal of clinical investigation, 69(3), pp. 617-631.

**Schobel, F., Jacobsen, I. D., and Brock, M**., 2010, "Evaluation of lysine biosynthesis as an antifungal drug target: biochemical characterization of Aspergillus fumigatus homocitrate synthase and virulence studies," Eukaryotic cell, 9(6), pp. 878-893.

**Schrettl, M., Beckmann, N., Varga, J., Heinekamp, T., Jacobsen, I. D., Jochl, C., Moussa, T. A., Wang, S., Gsaller, F., Blatzer, M., Werner, E. R., Niermann, W. C., Brakhage, A. A., and Haas, H.,** 2010, "HapX-mediated adaption to iron starvation is crucial for virulence of Aspergillus fumigatus," PLoS pathogens, 6(9), p. e1001124.

**Schrettl, M., Bignell, E., Kragl, C., Joechl, C., Rogers, T., Arst, H. N., Jr., Haynes, K., and Haas, H.,** 2004, "Siderophore biosynthesis but not reductive iron assimilation is essential for Aspergillus fumigatus virulence," J Exp Med, 200(9), pp. 1213-1219.

**Schrettl, M., Bignell, E., Kragl, C., Sabiha, Y., Loss, O., Eisendle, M., Wallner, A., Arst, H. N., Jr., Haynes, K., and Haas, H.,** 2007, "Distinct Roles for Intra- and Extracellular Siderophores during Aspergillus fumigatus Infection," PLoS Pathog, 3(9), p. e128.

**Schubert, M. S.,** 2009, "Allergic fungal sinusitis: pathophysiology, diagnosis and management," Medical mycology : official publication of the International Society for Human and Animal Mycology, 47 Suppl 1, pp. S324-330.

**Seagle, B. L., Gasyna, E. M., Mieler, W. F., and Norris, J. R., Jr.,** 2006, "Photoprotection of human retinal pigment epithelium cells against blue light-induced apoptosis by melanin free radicals from Sepia officinalis," Proceedings of the National Academy of Sciences of the United States of America, 103(45), pp. 16644-16648.

**Segal, B. H., and Romani, L. R.,** 2009, "Invasive aspergillosis in chronic granulomatous disease," Medical mycology : official publication of the International Society for Human and Animal Mycology, 47 Suppl 1, pp. S282-290.

**Seisenbaeva, G. A., Moloney, M. P., Tekoriute, R., Hardy-Dessources, A., Nedelec, J. M., Gun'ko, Y. K., and Kessler, V. G.,** 2010, "Biomimetic synthesis of hierarchically porous nanostructured metal oxide microparticles--potential scaffolds for drug delivery and catalysis," Langmuir : the ACS journal of surfaces and colloids, 26(12), pp. 9809-9817.

**Sheppard, D. C., Rieg, G., Chiang, L. Y., Filler, S. G., Edwards, J. E., Jr., and Ibrahim, A. S.,** 2004, "Novel inhalational murine model of invasive pulmonary aspergillosis," Antimicrob Agents Chemother, 48(5), pp. 1908-1911.

**Sheppard, D. C.,** 2011, "Molecular mechanism of Aspergillus fumigatus adherence to host constituents," Curr Opin Microbiol, 14(4), pp. 375-379.

**Singh, R., Jamieson, A., and Cresswell, P.,** 2008, "GILT is a critical host factor for Listeria monocytogenes infection," Nature, 455(7217), pp. 1244-1247.

**Slesiona, S., Gressler, M., Mihlan, M., Zaehle, C., Schaller, M., Barz, D., Hube, B., Jacobsen, I. D., and Brock, M.,** 2012, "Persistence versus escape: Aspergillus terreus and Aspergillus fumigatus employ different strategies during interactions with macrophages," PloS one, 7(2), p. e31223.

**Slesiona, S., Ibrahim-Granet, O., Olias, P., Brock, M., and Jacobsen, I. D.,** 2012, "Murine Infection Models for Aspergillus terreus Pulmonary Aspergillosis Reveal Long-term Persistence of Conidia and Liver Degeneration," Journal of Infectious Diseases, 205(8), pp. 1268-1277.

**Stanzani, M., Orciuolo, E., Lewis, R., Kontoyiannis, D. P., Martins, S. L., St John, L. S., and Komanduri, K. V.,** 2005, "Aspergillus fumigatus suppresses the human cellular immune response via gliotoxin-mediated apoptosis of monocytes," Blood, 105(6), pp. 2258-2265.

**Steele, C., Rapaka, R. R., Metz, A., Pop, S. M., Williams, D. L., Gordon, S., Kolls, J. K., and Brown, G. D.,** 2005, "The beta-glucan receptor dectin-1 recognizes specific morphologies of Aspergillus fumigatus," PLoS Pathog, 1(4), p. e42.

**Steinbach, W. J., Benjamin, D. K., Jr., Trasi, S. A., Miller, J. L., Schell, W. A., Zaas, A. K., Foster, W. M., and Perfect, J. R.,** 2004, "Value of an inhalational model of invasive aspergillosis," Medical mycology : official publication of the International Society for Human and Animal Mycology, 42(5), pp. 417-425.

**Stephens-Romero, S. D., Mednick, A. J., and Feldmesser, M.,** 2005, "The pathogenesis of fatal outcome in murine pulmonary aspergillosis depends on the neutrophil depletion strategy," Infect Immun, 73(1), pp. 114-125.

**Stergiopoulou, T., Meletiadis, J., Roilides, E., Kleiner, D. E., Schaufele, R., Roden, M., Harrington, S., Dad, L., Segal, B., and Walsh, T. J.,** 2007, "Host-dependent patterns of tissue injury in invasive pulmonary aspergillosis," American journal of clinical pathology, 127(3), pp. 349-355.

**Sturtevant, J. E., and Latge, J. P**., 1992, "Interactions between conidia of Aspergillus fumigatus and human complement component C3," Infect Immun, 60(5), pp. 1913-1918.

**Subira, M., Martino, R., Rovira, M., Vazquez, L., Serrano, D., and De La Camara, R.,** 2003,"Clinical applicability of the new EORTC/MSG classification for invasive pulmonary aspergillosis in patients with hematological malignancies and autopsy-confirmed invasive aspergillosis," Annals of hematology, 82(2), pp. 80-82.

**Susin, S. A., Daugas, E., Ravagnan, L., Samejima, K., Zamzami, N., Loeffler, M., Costantini, P., Ferri, K. F., Irinopoulou, T., Prevost, M. C., Brothers, G., Mak, T. W., Penninger, J., Earnshaw, W. C., and Kroemer, G.,** 2000, "Two distinct pathways leading to nuclear apoptosis," J Exp Med, 192(4), pp. 571-580.

**Suzuki, T., Nakanishi, K., Tsutsui, H., Iwai, H., Akira, S., Inohara, N., Chamaillard, M., Nunez, G., and Sasakawa, C.,** 2005, "A novel caspase-1/toll-like receptor 4-independent pathway of cell death induced by cytosolic Shigella in infected macrophages," The Journal of biological chemistry, 280(14), pp. 14042-14050.

**Szewczyk, E., and Krappmann, S**., 2010, "Conserved regulators of mating are essential for Aspergillus fumigatus cleistothecium formation," Eukaryotic cell, 9(5), pp. 774-783.

**Tarrand, J. J., Lichterfeld, M., Warraich, I., Luna, M., Han, X. Y., May, G. S., and Kontoyiannis, D. P.,** 2003, "Diagnosis of invasive septate mold infections. A correlation of microbiological culture and histologic or cytologic examination," American journal of clinical pathology, 119(6), pp. 854-858.

**Taylor, R. C., Cullen, S. P., and Martin, S. J.,** 2008, "Apoptosis: controlled demolition at the cellular level," Nat Rev Mol Cell Biol, 9(3), pp. 231-241.

**Tekaia, F., and Latge, J. P**., 2005, "Aspergillus fumigatus: saprophyte or pathogen?," Curr Opin Microbiol, 8(4), pp. 385-392.

**Thau, N., Monod, M., Crestani, B., Rolland, C., Tronchin, G., Latge, J. P., and Paris, S.,** 1994, "rodletless mutants of Aspergillus fumigatus," Infect Immun, 62(10), pp. 4380-4388.

**Thornberry, N. A., and Lazebnik, Y.,** 1998, "Caspases: enemies within," Science (New York, N.Y.), 281(5381), pp. 1312-1316.

**Thywissen, A., Heinekamp, T., Dahse, H. M., Schmaler-Ripcke, J., Nietzsche, S., Zipfel, P. F., and Brakhage, A. A.,** 2011, "Conidial Dihydroxynaphthalene Melanin of the Human Pathogenic Fungus Aspergillus fumigatus Interferes with the Host Endocytosis Pathway," Front Microbiol, 2, p. 96.

**Tournu, H., Serneels, J., and Van Dijck, P.,** 2005, "Fungal pathogens research: novel and improved molecular approaches for the discovery of antifungal drug targets," Curr Drug Targets, 6(8), pp. 909-922.

**Tronchin, G., Esnault, K., Renier, G., Filmon, R., Chabasse, D., and Bouchara, J. P.,** 1997, "Expression and identification of a laminin-binding protein in Aspergillus fumigatus conidia," Infect Immun, 65(1), pp. 9-15.

**Tsai, H. F., Chang, Y. C., Washburn, R. G., Wheeler, M. H., and Kwon-Chung, K. J.,** 1998, "The developmentally regulated alb1 gene of Aspergillus fumigatus: its role in modulation of conidial morphology and virulence," Journal of bacteriology, 180(12), pp. 3031-3038.

**Tsai, H. F., Washburn, R. G., Chang, Y. C., and Kwon-Chung, K. J.,** 1997, "Aspergillus fumigatus arp1 modulates conidial pigmentation and complement deposition," Mol Microbiol, 26(1), pp. 175-183.

**van der Wel, N., Hava, D., Houben, D., Fluitsma, D., van Zon, M., Pierson, J., Brenner, M., and Peters, P. J.,** 2007, "M. tuberculosis and M. leprae translocate from the phagolysosome to the cytosol in myeloid cells," Cell, 129(7), pp. 1287-1298.

**van Duin, D., Casadevall, A., and Nosanchuk, J. D.,** 2002, "Melanization of Cryptococcus neoformans and Histoplasma capsulatum reduces their susceptibilities to amphotericin B and caspofungin," Antimicrob Agents Chemother, 46(11), pp. 3394-3400.

**VandenBergh, M. F., Verweij, P. E., and Voss, A**., 1999, "Epidemiology of nosocomial fungal infections: invasive aspergillosis and the environment," Diagnostic microbiology and infectious disease, 34(3), pp. 221-227.

**Vennstrom, L., Bysell, C., Bjorkelund, H., Lundqvist, H., and Andersson, K.,** 2008, "Real-time viability assay based on 51Cr retention in adherent cells," BioTechniques, 44(2), pp. 237-240.

**Verweij, P. E., and Denning, D. W.,** 1997, "The challenge of invasive aspergillosis: increasing numbers in diverse patient groups," International Journal of Infectious Diseases, 2(2), pp. 61-63.

**Viollier, A. F., Peterson, D. E., De Jongh, C. A., Newman, K. A., Gray, W. C., Sutherland, J. C., Moody, M. A., and Schimpff, S. C.,** 1986, "Aspergillus sinusitis in cancer patients," Cancer, 58(2), pp. 366-371.

**Volling, K., Brakhage, A. A., and Saluz, H. P.,** 2007, "Apoptosis inhibition of alveolar macrophages upon interaction with conidia of Aspergillus fumigatus," FEMS Microbiol Lett, 275(2), pp. 250-254.

**Volling, K., Thywissen, A., Brakhage, A. A., and Saluz, H. P.,** 2011, "Phagocytosis of melanized Aspergillus conidia by macrophages exerts cytoprotective effects by sustained PI3K/Akt signalling," Cell Microbiol, 13(8), pp. 1130-1148.

**Wang, X., and Albertioni, F.,** 2010, "Effect of clofarabine on apoptosis and DNA synthesis in human epithelial colon cancer cells," Nucleosides, nucleotides & nucleic acids, 29(4-6), pp. 414-418.

**Wang, Y., Aisen, P., and Casadevall, A.,** 1995, "Cryptococcus neoformans melanin and virulence: mechanism of action," Infect Immun, 63(8), pp. 3131-3136.

**Warnock, G., Turtoi, A., Blomme, A., Bretin, F., Bahri, M. A., Lemaire, C., Libert, L. C., Seret, A. E., Luxen, A., Castronovo, V., and Plenevaux, A. R.,** 2013, "In vivo PET/CT in a human glioblastoma chicken chorioallantoic membrane model: a new tool for oncology and radiotracer development," Journal of nuclear medicine : official publication, Society of Nuclear Medicine, 54(10), pp. 1782-1788.

**Washburn, R. G., Kennedy, D. W., Begley, M. G., Henderson, D. K., and Bennett, J. E.,** 1988, "Chronic fungal sinusitis in apparently normal hosts," Medicine, 67(4), pp. 231-247.

**Wasylnka, J. A., and Moore, M. M.,** 2002, "Uptake of Aspergillus fumigatus Conidia by phagocytic and nonphagocytic cells in vitro: quantitation using strains expressing green fluorescent protein," Infect Immun, 70(6), pp. 3156-3163.

**Wasylnka, J. A., and Moore, M. M.,** 2003, "Aspergillus fumigatus conidia survive and germinate in acidic organelles of A549 epithelial cells," J Cell Sci, 116(Pt 8), pp. 1579-1587.

**Weeks, A. J., Jauregui-Osoro, M., Cleij, M., Blower, J. E., Ballinger, J. R., and Blower, P. J.,** 2011, "Evaluation of [18F]-tetrafluoroborate as a potential PET imaging agent for the human sodium/iodide symporter in a new colon carcinoma cell line, HCT116, expressing hNIS," Nuclear medicine communications, 32(2), pp. 98-105.

**Weidner, G., d'Enfert, C., Koch, A., Mol, P. C., and Brakhage, A. A.,** 1998, "Development of a homologous transformation system for the human pathogenic fungus Aspergillus fumigatus based on the pyrG gene encoding orotidine 5'-monophosphate decarboxylase," Curr Genet, 33(5), pp. 378-385.

**Welling, M. M., Paulusma-Annema, A., Balter, H. S., Pauwels, E. K., and Nibbering, P. H., 2000**, "Technetium-99m labelled antimicrobial peptides discriminate between bacterial infections and sterile inflammations," European journal of nuclear medicine, 27(3), pp. 292-301.

**Williams, D. M., Weiner, M. H., and Drutz, D. J.,** 1981, "Immunologic studies of disseminated infection with Aspergillus fumigatus in the nude mouse," The Journal of infectious diseases, 143(5), pp. 726-733.

**Williams, G. T.,** 1994, "Programmed cell death: a fundamental protective response to pathogens," Trends in microbiology, 2(12), pp. 463-464.

**Wingard, J. R., Beals, S. U., Santos, G. W., Merz, W. G., and Saral, R**., 1987, "Aspergillus infections in bone marrow transplant recipients," Bone marrow transplantation, 2(2), pp. 175-181.

**Wurbach, L., Heidrich, A., Opfermann, T., Gebhardt, P., and Saluz, H. P.,** 2012, "Insights into bone metabolism of avian embryos in ovo via 3D and 4D 18F-fluoride positron emission tomography," Molecular imaging and biology : MIB : the official publication of the Academy of Molecular Imaging, 14(6), pp. 688-698.

**Yang, Z., Kontoyiannis, D. P., Wen, X., Xiong, C., Zhang, R., Albert, N. D., and Li, C., 2009**, "Gamma scintigraphy imaging of murine invasive pulmonary aspergillosis with a (111)In-labeled cyclic peptide," Nuclear medicine and biology, 36(3), pp. 259-266.

**Youngchim, S., Morris-Jones, R., Hay, R. J., and Hamilton, A. J.,** 2004, "Production of melanin by Aspergillus fumigatus," Journal of medical microbiology, 53(Pt 3), pp. 175-181.

**Yu, D., Jin, C., Leja, J., Majdalani, N., Nilsson, B., Eriksson, F., and Essand, M.,** 2011, "Adenovirus with hexon Tat-protein transduction domain modification exhibits increased therapeutic effect in experimental neuroblastoma and neuroendocrine tumors," Journal of virology, 85(24), pp. 13114-13123.

**Zhong, Y., Weininger, M., Pirbhai, M., Dong, F., and Zhong, G**., 2006, "Inhibition of staurosporine-induced activation of the proapoptotic multidomain Bcl-2 proteins Bax and Bak by three invasive chlamydial species," The Journal of infection, 53(6), pp. 408-414.

**Zhou, Y., and Liu, S.,** 2011, "$^{64}$Cu-labeled phosphonium cations as PET radiotracers for tumor imaging," Bioconjugate chemistry, 22(8), pp. 1459-1472.

# Abbreviations

| | |
|---|---|
| % | Percentage |
| °C | Degree Celsius |
| Δ | Deletion |
| α | Alpha |
| β | Beta |
| **A** | |
| *A*. | *Aspergillus* |
| ABPA | Allergic bronchopulmonary aspergillosis |
| AMM | *Aspergillus* minimal medium |
| APS | Ammonium persulfate |
| Arp1 | Aspergillu reddish-pink 1 |
| Arp2 | Aspergillus reddish-pink 2 |
| Ayg1 | Aspergillus yellowish-green 1 |
| **B** | |
| BSA | Bovine serum albumin |
| bp | base pair |
| **C** | |
| CAM | chorioallantoic membrane |
| casp | Caspase |
| CFU | Colony-forming units |
| CHX | cycloheximide |
| CGD | chronic granulomatous disease |
| $CO_2$ | carbon dioxide |
| cm | centimeter |
| CPS | counts per second |
| CT | Computed Tomography |
| **D** | |
| DHN | Dihydroxynaphthalene |
| DMSO | Dimethylsulfoxide |
| DNA | Deoxyribonucleic acid |
| DOPA | Dihydroxyphenylalanine |
| **E** | |
| e.g. | For example |
| EDTA | Ethylenediaminetetraacetic acid |
| **F** | |
| FACS | Fluorescene activated cell sorting |
| FBS | Fetal bovine serum |
| FDG | 2-[$^{18}$F]fluoro-2-deoxyglucose |
| Fig | Figure |
| FITC | Fluorescein isothiocyanate |
| **G** | |
| g | gram or acceleration of gravity |
| $^{68}$Ga | Gallium-68 |
| GM | Galactomannan test |
| **H** | |
| h | hour |
| HCl | hydrochloric acid |
| HE | Hematoxylin eosin (stain) |
| **I** | |
| IA | Invasive aspergillosis |
| ID | Injected dose |
| i.e. | That is |
| i.p. | intra peritoneal |
| IL | Interleukin |
| **K** | |
| kDa | kilo dalton |
| **L** | |
| LPS | Lipo polysaccharide |
| L | Litre |
| **M** | |
| M | Molar |
| MG | Melanin ghosts |
| µ | micro |
| µg | microgram |
| min | minute (s) |
| ml | millilitre |
| µl | microlitre |
| µm | micrometre |
| mM | milliMolar |
| MOI | Multiplicity of Infection |
| **N** | |
| n | number |
| NaCl | sodium chloride |
| **P** | |
| PAA | polyacrylamide |
| PAS | periodic acid Schiff |
| PBS | phosphate buffer saline |
| PC | phosphte citrate |
| PCR | polymerase chain reaction |
| PFA | paraformaldehyde |

| | |
|---|---|
| PAMP | pathogen associated molecular pattern |
| PET | Positron Emission Tomography |
| p.i. | post infection |
| pksP | polyketide synthase |
| PRR | pathogen recognition receptors |
| PVDF | polyvinylidene sifluoride |
| **R** | |
| Rpm | rounds per minute |
| RPMI | Rosewell Park Memorial Institute |
| rRNA | ribosomal ribonucleic acid |
| ROI | region of interest or reactive oxygen intermediates |
| ROS | reactive oxygen species |
| RT | room temperature |
| **S** | |
| s.d. | standard deviation |
| sec | second |
| SDS | sodium dodecyl sulphate |
| spp. | species (plural) |
| STS | staurosporine |
| **T** | |
| Tab. | table |
| TAFC | triacetyl fuarinine C |
| TEMED | N,N,N,N-Tetra methylethylenediamine |
| TLR | Toll like receptor |
| TNF | tumor necrosis factor |
| **V** | |
| v/v | volume/volume |
| **W** | |
| w/v | weight/volume |
| wo | without |
| wt | wild type |
| **Z** | |
| Z-VAD-FMK | carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone |

# Curriculum vitae

## Personal Data

| | |
|---|---|
| Name | Shayista Amin |
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## Educational qualification

| | |
|---|---|
| **2010 - 2014** | Doctoral research student<br>Leibniz Intitute for Natural Product Research and Infection Biology – Hans-Knöll-Institute, Jena.<br>Department of Cell and Molecular Biology<br>Prof. Dr. Hans Peter Saluz<br><br>Title: "*In vitro*, *in ovo* and *in vivo* interactions of human pathogenic fungi *Aspergillus fumigatus*"<br><br>Member of "International Leibniz Research School for Microbial and Biomolecular Interactions (ILRS)" |
| **2009** | Post Master thesis<br>Dr. B. R. Ambedkar Center for Biomedical Research, University of Delhi, India.<br><br>Title: "Effect of LOX inhibitor (Baicalein) on hypobaric hypoxia-induced memory dysfunction" |
| **2006 - 2008** | Master`s in Mirobiology<br>The Oxford College of Science, Bangalore University, India |
| **2002 - 2005** | Bachelor`s in Science<br>(Zoology, Botany, Chemistry English)<br>Govt. College for Women, University of Kashmir, India |
| **2000 – 2001** | Higher Secondary School (XII)<br>Board of School Education. Jammu & Kashmir, India |
| **1999** | Higher Secondary School (X)<br>Board of School Education, Jammu & Kashmir, India |

# List of publications

**Parts of this thesis are included in the following publications:**

**Amin S.,** Thywissen A, Heinekamp T**,** Saluz HP**,** Brakhage AA (2014)

Melanin dependent survival of Aspergillus fumigatus conidia in lung epithelial cells.

International Journal of Medical Microbiology, 10:1016 (Epub ahead of print)

**Parts of this thesis were presented at the following scientific conferences:**

**Amin, S.**, Opfermann, T., Gebhart, P., Brakhage A.A., Saluz, HP. (2011) oral presentation

Molecular Imaging as tool for tracing host-pathogen interaction between *Aspergillus fumigatus* and chick embryos.

ILRS symposium, Dornburg, Germany.

**Amin, S.**, Heinekemp, T., Brakhage, A.A., Saluz, HP. (2012) oral presentation

Interaction of *Aspergillu fumigatus* with lung epithelial cells.

ILRS symposium, Rosensäle, FSU, Jena.

**Amin, S.**, Heinekemp, T., Brakhage, A.A., Saluz, HP. (2012) poster presentation

Interaction of *Apergillus fumigatus* with lung epithelial cells.

Annual Scientific Meeting of Britih Society for Medical Mycology, Cardiff, UK.

**Amin, S.**, Heinekemp, T., Brakhage, A.A., Saluz, HP. (2012) poster presentation

Interaction of *Aspergillus fumigatus* with lung epithelial cells: role of melanin

International Society for Human and Animal Mycology (ISHAM 2012), Berlin, Germany.

**Amin, S.**, Heinekemp, T., Brakhage, A.A., Saluz, HP. (2013) poster presentation

Interaction of *Aspergillus fumigatus* with lung epithelial cells: role of melanin

ILRS symposium, HKI, Jena.

**Amin, S.**, Heinekemp, T., Brakhage, A.A., Saluz, HP. (2013) poster presentation

Interaction of *Aspergillus fumigatus* with lung epithelial cells and role of melanin

Microbial Pathogenesis and Host Response, Cold Spring Harbor, New York, USA.

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## Declaration

I hereby declare that the work presented in this thesis entitled

"*In vitro*, *in vivo* and *in ovo* interactions of human pathogenic fungus *Aspergillus fumigatus*"

is my own and has been produced by me as a result of my own original research. This research has not been previously accepted for any degree and is not being currently considered for any other degree at any other university. Every effort is made to indicate the contributions of others wherever involved, with due reference to the literature and acknowledgement of collaborative discussions and research.

…….. ……

Jena Shayista Amin